UNIVERSITY OF LONDON THESIS

Degree PhD Year 2007 Name of Author PAUL STUART REDFORD

Regulatory mechanisms inhibiting anti-mycobacterial immunity following *Mycobacterium tuberculosis* infection.

Paul Stuart Redford

Division of Immunoregulation,
National Institute for Medical Research,
The Ridgeway, Mill Hill,
London, NW7 1AA.

A thesis presented for the degree of Doctor of Philosophy in the University of London, 2007.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I Paul Stuart Redford confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

16/3/07

Abstract:

The work reported in this thesis addresses the regulatory factors that function to limit the initiation of protective immune responses following exposure to the bacterium *Mycobacterium tuberculosis* (MTb). Control and clearance of intracellular pathogens, such as MTb, is dependent on the cytokine Tumour Necrosis Factor (TNF) and induction of a T-helper 1 (Th1) response, which is characterised by production of IFN-gamma driven by interleukin (IL)-12. In other infection models the presence of the immunosuppressive cytokine IL-10 in the local milieu has been shown to down-regulate Th1 responses thus limiting detrimental host induced immune-pathology.

To determine a role for IL-10 following murine infection, we examined its function during acute and chronic infections with two strains of H37Rv obtained from either *i)* National Institute for Medical Research (NIMR) or *ii)* London School of Hygiene and Tropical Medicine (LSHTM). IL-10 receptor blockade during the chronic phase of MTb infection reduced bacterial burdens in mice infected with H37Rv NIMR, but not mice infected with H37Rv LSHTM. However, despite the lack of effect of IL-10 blockade on the bacterial load during chronic infection with H37Rv LSHTM, immune cells obtained from MTb infected mice produced elevated levels of IFN-gamma when stimulated *in vitro* in the presence of IL-10 blocking antibodies. In addition, neutralisation of IL-10 before and during acute MTb infection with H37Rv LSHTM resulted in a transient reduction in bacterial burdens and enhanced IFN-gamma production, suggesting that IL-10 plays a role in regulating the early immune response to MTb.

Additional regulators that may function together with or in parallel to IL-10 to limit bacterial clearance such as regulatory T cells (Tregs) have been shown to be

regulators of autoimmunity, atopy and infectious disease. Using flow cytometric analysis of the Treg specific transcription factor FoxP3, we observed an early increase in the number of lung Tregs following aerosol MTb infection of mice. However, when addressing the effect on bacterial clearance in the absence of Tregs by either *i)* antibody depletion or *ii)* adoptive transfer approaches into immuno-deficient mice, a suppressive role for Tregs on bacterial burdens could not be found.

Finally this work evaluated the role of plasmacytoid precursor DC (pDC) during MTb infection, which is in contrast their normal function as mediators of the anti-viral response. Upon *in vitro* exposure to viable MTb, plasmacytoid pDC could not be infected and did not produce pro-inflammatory cytokines. Using flow cytometry, we observed no increase in plasmacytoid pDC in either the lung or spleen during the early stages of aerosol or intravenous infection. In addition, antibody depletion of plasmacytoid pDC during the early stages of MTb infection did not affect bacterial load. In summary, the data suggests that plasmacytoid pDC play only a minor role during the early immune response to MTb.

Acknowledgements:

Firstly, I would like to thank Anne O' Garra my primary supervisor for giving me the opportunity to obtain my PhD in such a prestigious lab. I would also like to thank her for insightful meetings, her endless drive, support and ambition during some testing times through my PhD career. Discussions with Anne either in her office, in the corridor, or on the phone from Mexico, still leave me in awe as she possesses an extremely rare ability to regurgitate data (either published, from conversations at scientific meetings, the pub/bar or other), from any aspect of immunology over the last 20 years with incredible accuracy.

I would like to also thank Greg Bancroft, my second supervisor, for agreeing to take me on as a student following the sad death of M. Jo Colston during the early stages of my PhD. Greg has been an influential figure with regard to discussions on the variability between H37Rv strains used in our *in vivo* MTb studies and without the generous un-restricted use of some of the BSL-3 facilities at the LSHTM, certain aspects of my work would not have been possible.

During my PhD I was given the opportunity to work closely with Andre Boonstra, a very talented post-doc within the AO'G lab. Interacting so closely with someone on a day-to-day basis could have been a testing experience, but working with Andre was truly enjoyable and insightful; this collaboration provided me with a solid basis in order to initiate my scientific career. Cheers mate for everything!

Thanks also go to the all the members of the AO'G lab for making my PhD such a great experience. Special thanks must go to:

Mary Holman - for her steady I.V. hands, early morning coffee discussions and the best muffins and banana bread I have ever tasted.

Simon Read - thanks for a great start to a new MTb collaboration.

Margarida Saraiva - for teaching me about Portugal, Port wine and Jose Mourinho.

I would especially like to say a big thank you to my partner Lisa, who over the last 3 years has become the most influential person in my life. I can't thank her enough for all her love, support and gorgeous food when I was to tired to cook.

Last but no means least I would like to thank my parents, Sheila and Dennis, for all their time, endless love and support as without it I would not be where I am today, thank-you!

Table of Contents:

Table of Figures:

Chapter 1: Introduction.

Chapter 3: The Role of IL-10 in Regulating Protective Immunity to MTb Infection.

Chapter 4: The Potential Role for Naturally Occurring Regulatory T cells As Mediators of MTb Survival and Persistence.

Chapter 5: The Role of Plasmacytoid pDC in Anti-Mycobacterial Immunity.

Table of Tables:

List of Abbreviations:

AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
BCG	bacille Calmette-Guerin
BM	bone marrow
BSL-3	biological safety level-3
CD	cluster of differentiation
CFP-10	culture filtrate protein-10
CFU	colony forming unit
CpG	cytosine-phosphodiester-guanine
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
DHM	didehydroxymycobactin
DNA	deoxyribonucleic acid
EAE	experimental allergic encephalomyelitis
ESAT-6	6 kDa early secretory antigenic target
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Flt-3L	FMS like tyrosine kinase-3 ligand
FoxP3	forkhead box P3
GM-CSF	Granulocyte/monocyte colony stimulating factor
HIV	human immuno-deficiency virus
HSV	herpes simplex virus
IFN	interferon

IL-	interleukin
iNK	invariant natural killer cells
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
IPEX	immune dysregulation, polyendocrinopathy enteropathy, X linked
IV	intravenous
KO	knockout
LAM	lipoarabinomannan
LN	lymph node
LPS	lipopolysaccharide
LSHTM	London School of Hygiene an Tropical Medicine
MACS	magnetic cell sorting
MCP-1	monocyte chemo-attractant protein-1
MDR-TB	multi-drug resistant tuberculosis
MHC	major histo-compatibility complex
MOI	multiplicity of infection
MTb	Mycobacterium tuberculosis
NIMR	National Institute for Medical Research
NK	natural killer
OADC	oleic acid-albumin dextrose catalase
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PPD	purified protein derivative
pDC	precursor dendritic cell

PIM	phosphatidylinositol mannosidase
RNA	ribonucleic acid
RNI	reactive nitrogen intermediate
TB	tuberculosis
Th	T helper cell
TGF	transforming growth factor
TIRAP	Toll IL-1 receptor associated protein
TLR	toll-like receptor
TNF	tumour necrosis factor
TRAM	TRIF related adaptor molecule
Tregs	regulatory T cells
TRIF	TIR domain containing adaptor protein inducing interferon.

Chapter 1: Introduction.

1.1. *Mycobacterium tuberculosis* (MTb):

Robert Koch first identified MTb as the causative agent of tuberculosis (TB) in 1882, to date, TB is once more on the increase and is responsible for approximately 8 million new cases and 2 million deaths annually (WHO, 2006). The incidence of TB is commonly associated with developing economies such as those in sub-Saharan Africa, however, the number of reported cases in developed economies such as the UK (Fig. 1.1A), has seen a dramatic 10% increase from 2004 (7,240 cases) to 2005, (8,027 cases) with the vast majority of cases occurring in London (Fig. 1.1B) (Health Protection Agency UK (HPA, 2006).

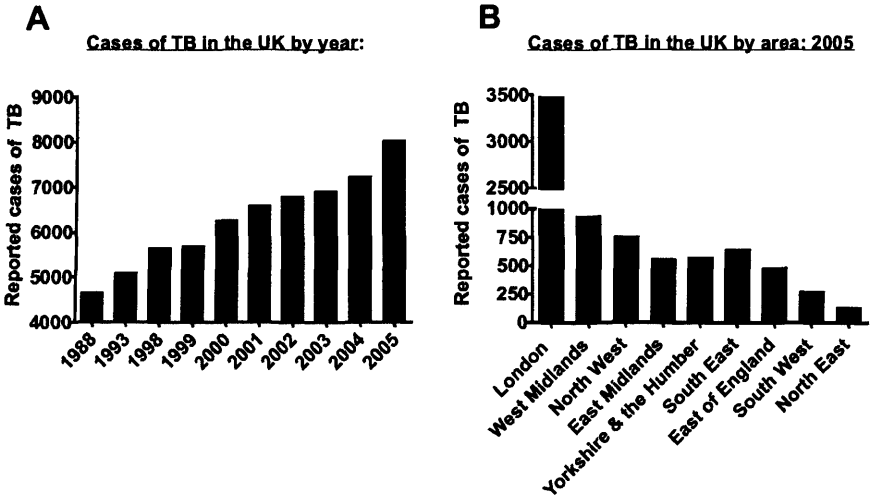


***Figure 1.1**: The number of reported cases of TB are on the increase in the UK and more specifically in London.* **(Data adapted from the HPA-UK, 2006).**

On average, 90% of individuals exposed to MTb mount an efficient cell mediated immune response culminating in a latent infection with no visible signs of disease. The remaining 10% of individuals for reasons that are at not fully understood, will progress to active disease as demonstrated by culturable bacilli from the sputum

(Kaufmann, 2001, Frieden *et al.*, 2003). Over the past ten years TB has been on the increase worldwide. In 2000, TB was the cause of 11% of deaths in individuals co-infected with HIV/AIDS and to date, in South Africa alone more than 2 million people are co-infected with TB and HIV/AIDS (North *et al.*, 2004). Globally it is believed that of the 45 million people infected with HIV/AIDS, approximately 15 million are co-infected with TB (WHO, 2006). The close association of TB and HIV/AIDS highlights the importance of CD4+ T cells in mediating anti-MTb immunity.

1.2. Artillery in the fight against TB:

1.2.1. BCG:

In 1921, the development of bacille Calmette-Guerin (BCG) was heralded as a major breakthrough in the fight against TB and to date still is the only current licensed vaccine for TB. The BCG vaccine was derived from *in vitro* passaging of *Mycobacterium bovis*, the *Mycobacterium* responsible for bovine TB, on potato slices soaked in ox gall (Kaufmann, 2006a). During the passaging process key genes of stimulatory molecules were shown to have been lost from BCG, such as the T cell antigens ESAT-6 and CFP-10 from mutations in the BCG *RD1* gene (Pym *et al.*, 2002), these antigens are readily abundant in live virulent MTb (Harboe *et al.*, 1996). It has been estimated that more than 3 billion doses of BCG have been administered to date globally. (WHO, 2006). In the UK, the vaccination of school children over a period of 15 years has led to sharp 70% decline in the incidence of TB (Young *et al.*, 2006). To date, BCG shows great efficacy in preventing childhood meningeal TB, but shows extensive variability in protection from adult pulmonary TB that is

suggested to be dependent on geographical location (Fine, 1995). The reasoning for this variation in the efficacy of BCG globally is not fully understood, however, it is believed that the pre-existing immune response in vaccinated individuals to shared antigens between mycobacterial species and MTb may result in sub-optimal vaccination (Andersen *et al.*, 2005).

1.2.2. Antibiotics and drug resistance:

The advent of antibiotics including streptomycin in 1943 was seen as a major breakthrough in the battle against TB. The further discovery of Isoniazid, Pyrazinamide, Ethambutol and Rifampin during 1950-1960 led to a decline in the number of cases of TB. However it is believed that during the late 1970's and early 1980's the cessation of treatment regimens including the closure of sanatoriums in developed countries, led to a resurgence in the number of cases until 1993 when the WHO declared TB a global emergency (WHO, 1993).

Patients displaying symptoms of active TB can be treated with a cocktail of front-line antibiotics - Isoniazid, Ethambutol, Pyrazinamide and Rifampin (Wright, 2006). Although antibiotic chemotherapy is effective in reducing infectivity, treatment lasts for at least six months, gives severe side effects e.g. liver damage, and does not achieve sterile cure. In addition, the increasing failure for patients to comply with the long and arduous drug regimen often results in multi-drug resistant forms of TB (MDR-TB) (Kaufmann, 2001, WHO, 2006, Wright, 2006). The incidence of MDR-TB has been on the increase since the late 1980's but in contrast to antibiotic resistance associated by horizontal gene transfer, as was the case for penicillin, drug

resistance in MTb is associated with chromosome mutations (Young, 2003). Patients with MDR-TB, if treatable, receive an extensive course of chemotherapy consisting of multiple first and second-line antibiotics lasting up to two years. In latently infected individuals or in patients that have completed chemotherapy, the TB bacillus can persist for years without giving any symptoms (Kaufmann, 2006b). Now in the 21st century, the increasing rise in cases of TB but more prominently the emergence of multiple drug resistant strains of MTb, the rising incidence in HIV/AIDS and air travel between continents means the spreading and lethality of MTb has never been greater.

1.3. An overview of the immune response and its role in eradicating invading pathogens:

The maintenance of immune homeostasis involves the concise interplay between a multitude of immune cells and their secreted biologically active soluble factors. Their aim is to function harmoniously in order to regulate autoimmunity, atopy or eradicate infection whilst minimising host immune-pathology. The key players in the immune system and more specifically with respect to mediating immunity to MTb infection, will be discussed below:

1.3.1. Macrophages:

1.3.1.1. Background:

Macrophages originate from circulating bone marrow derived monocytes and play a pivotal role in the innate immune response to infection by ingesting, controlling and eradicating intracellular pathogens such as *Listeria* (Portnoy *et al.*, 2002),

Leishmania (Engwerda *et al.*, 2004), *Toxoplasma gondii* (Gazzinelli *et al.*, 1992) and MTb (North *et al.*, 2004) through the production of anti-microbial reactive oxygen and nitrogen intermediates (Shiloh *et al.*, 2000). Macrophages can be derived from distinct sub-populations in lymphoid organs, but in addition can also be obtained from non-lymphoid tissues such as the lung, liver and epidermis (Taylor *et al.*, 2005). Macrophages are normally resident at natural sites of pathogen entry e.g. the lung, and therefore express a large variety of pathogen recognition receptors to facilitate the recognition of invading organisms or their products and mount an appropriate immune response (Taylor *et al.*, 2005). Following stimulation with the toll-like receptor (TLR) ligands LPS (TLR-4) and CpG-DNA (TLR-9), macrophages produce Tumour Necrosis Factor (TNF), interleukin (IL)-12p40, IL-6 as well as the anti-inflammatory cytokine IL-10 (Boonstra *et al.*, 2006).

1.3.1.2. Macrophages and MTb:

The importance of macrophages in the immune response to MTb has been well documented (Armstrong *et al.*, 1975, Schlesinger, 1993, Gomes *et al.*, 1999, Underhill *et al.*, 1999, Gonzalez-Juarrero *et al.*, 2003). Upon inhalation of MTb bacilli, the first lines of defence are resident lung alveolar macrophages. Macrophages readily phagocytose the MTb bacteria and become activated producing the cytokines TNF and IL-12 (Kaufmann, 2001). Once phagocytosed, the MTb bacillus survives and persists inside the macrophage by inhibition of phagosome-lysosome fusion (Armstrong *et al.*, 1971), in doing this MTb can still gain access to essential nutrients, e.g. iron, via the early endocytic pathway (Sturgill-Koszycki *et al.*, 1996, Collins *et al.*, 2002). Cytokine activation of macrophages by interferon (IFN)-gamma leads to enhanced production of reactive oxygen and nitrogen

intermediates that mediate mycobacterial killing (Chan *et al.*, 1992, Chan *et al.*, 1995, Flynn *et al.*, 1998, MacMicking *et al.*, 1997). Furthermore, activation of macrophages by IFN-gamma but not MTb, is dependent on the intracellular signalling adaptor molecule MyD88 (Shi *et al.*, 2003).

1.3.2. Dendritic cells (DC):

1.3.2.1. Background:

DC act as sentinels for pathogens, migrating through the blood to peripheral tissue to uptake antigens, then migrating to the lymph nodes and spleen, and processing and presenting antigens to T cells thus initiating immune responses (Banchereau, 1998, Lanzavecchia *et al.*, 2001, Steinman, 1991, Shortman *et al.*, 2002). DC can be derived from the bone marrow (BM) after culture resulting in myeloid DC and plasmacytoid pDC. In the mouse, both can produce IL-12 and TNF upon triggering through appropriate receptors but only plasmacytoid pDC produce IFN-alpha in response to viral infection. DC can also be directly isolated from the spleen as classical DC (CD8a+ or CD8a-, CD11b+ DC) or splenic plasmacytoid pDC (Shortman *et al.*, 2002). The flexibility of different DC in response to pathogens and other environmental signals enables them to direct Th1 or Th2 responses (Fig. 1.2) (Boonstra *et al.*, 2003, Kapsenberg, 2003). In response to microbial stimulus DC and macrophages produce bioactive IL-12 (more specifically IL-12p70), a cytokine that comprises of two subunits, a 35kDa light chain (p35) and a 40 kDa heavy chain (p40). It has been shown that the IL-12 (p40) subunit can also associate with another molecule called p19, to form a heterodimeric cytokine called IL-23, a key mediator involved in activation of long lived memory T cells (Trinchieri, 2003). Following

microbial stimulus IL-12 has a polarising effect on CD4+ T cells driving them towards a Th1 phenotype (Fig. 1.2), characterised by production of IFN-gamma (Robinson *et al.*, 2002, Trinchieri, 2003). In contrast, the immunosuppressive cytokine IL-10 can dampen Th1 responses by directly acting on the DC and macrophages to inhibit the production of IL-12 (Fiorentino *et al.*, 1991b). Th1 responses are in contrast to Th2 responses, that are categorised by IL-4, IL-5, IL-13 (Sher *et al.*, 1992, O'Garra, 1998) promoting protection against extracellular pathogens such as filarial worms (Whelan *et al.*, 2000).

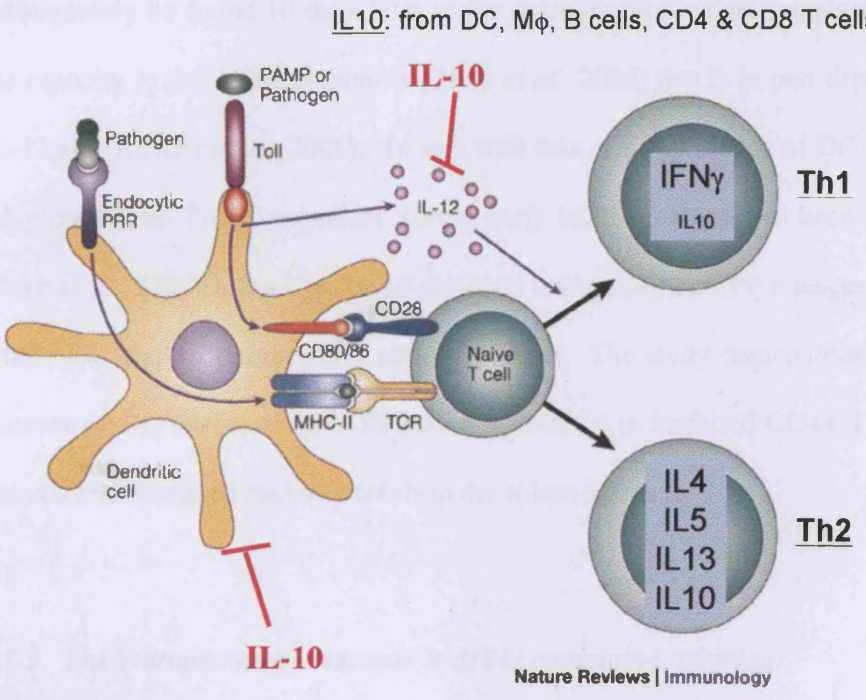


***Figure 1.2*: IL-12 production by DC following TLR ligation results in the development of Th1 cells.** (**Medzhitov, 2001**).

1.3.2.2. DC and MTb:

Similar to macrophages, DC can phagocytose MTb bacilli (Henderson *et al.*, 1997, Gonzalez-Juarrero *et al.*, 2001a) and become activated leading to the production of the cytokines TNF and IL-12 (Bodnar *et al.*, 2001, Giacomini *et al.*, 2001). In contrast to macrophages, DC are not able to kill intracellular MTb (Bodnar *et al.*, 2001, Tailleux *et al.*, 2003). However, DC play a key role in the uptake of MTb (Garcia-Romo *et al.*, 2004) and BCG (Humphreys *et al.*, 2006), then migrate to local draining lymph nodes. It has also been reported that following instillation of DC (pre-treated with heat-killed MTb *in vitro*) into the trachea of naïve mice, DC can subsequently be found 18 days later in the draining mediastinal lymph nodes with the capacity to drive Th1 responses (Bhatt *et al.*, 2004) that is in part dependent on IL-12p40 (Khader *et al.*, 2006). In line with this, the importance of DC in driving adaptive CD4+ T cell responses during early MTb infection has been shown by (Tian *et al.*, 2005) using a pCD11c-diptheria toxin receptor/GFP transgenic mouse, where DC could be transiently ablated *in vivo*. The study demonstrated that the absence of DC during early MTb infection resulted in impaired CD4+ T cell IFN-gamma and increased bacterial levels in the spleen and lungs.

1.3.3. The granulomatous response to MTb: containing infection:

Following macrophage phagocytosis of invading MTb bacilli, a host-pathogen standoff occurs where a small percentage of MTb bacilli survive by evading the anti-microbial mechanisms of the macrophage and persist within the un-fused phagosomes giving rise to a latent infection (Armstrong *et al.*, 1971). The composition of the granuloma in human pulmonary TB has to date received little

attention since original reports by the classical pathologists at the turn of the 20th century (Ulrichs *et al.*, 2006). In contrast, animal models such as the mouse and guinea pig have been the subject of intense investigation as MTb infection in animals slowly progresses to a disease that is both physiologically and immunologically similar to human TB.

1.3.3.1. The granulomatous response in human MTb:

Granulomas in human MTb infection can be subdivided into *a)* caseating (tuberculoid) granulomas (Fig. 1.3) or *b)* non-caseating (sarcoid) granulomas (Fig. 1.4). Following inhalation of MTb bacilli, a local inflammatory response mediated by infected alveolar macrophages and epithelial cells, gives rise to the formation of granulomas where bacilli are controlled and contained (Fayyazi *et al.*, 2000). If protective immunity is impaired in the infected host, tuberculoid granulomas may develop. These contain a soft to firm "cheese-like" caseated centre (hence the Latin name *caseous detritus*) comprising largely of host cell debris, bacterial and host derived lipids, necrotic macrophages and fused infected epithelial cells or giant cells (Fig.1.3) (Kaufmann, 2001). Due to the caseated composition of tuberculoid granulomas, they contain large numbers of infectious bacilli that can be released in aerosol droplets by coughing in order to spread infection. However, in mice caseating tuberculoid granulomas do not occur (Co *et al.*, 2004). Studies in human TB patients have identified the presence of IFN-gamma and IL-12p40 in sarcoid granulomas (Fenhalls *et al.*, 2002). Interestingly, granulomas progressing towards a tuberculoid phenotype (or tuberculoid granulomas) were commonly associated with the production of TNF but not IFN-gamma, implying a down-modulation of T cell responses at this stage (Fenhalls *et al.*, 2000).

More recently, the presence of peripheral structures that closely resemble lymphoid follicles have been reported in close proximity to granulomas (Ulrichs *et al.*, 2004). The peripheral follicles were located close to blood/lymphatic vessels and comprised predominantly of infected antigen presenting cells (APC), and B cells with few CD4+ and CD8+ T cells. The authors hypothesise that the presence of a peripheral lymphoid structure is to serve as a hub in order to co-ordinate the ongoing immune response to MTb. The composition of peripheral lymphoid follicles is in contrast to the main granuloma which comprises predominantly of infected APC and CD4+ T cells with relatively low numbers of peripheral CD8+ T cells (Ulrichs *et al.*, 2004).

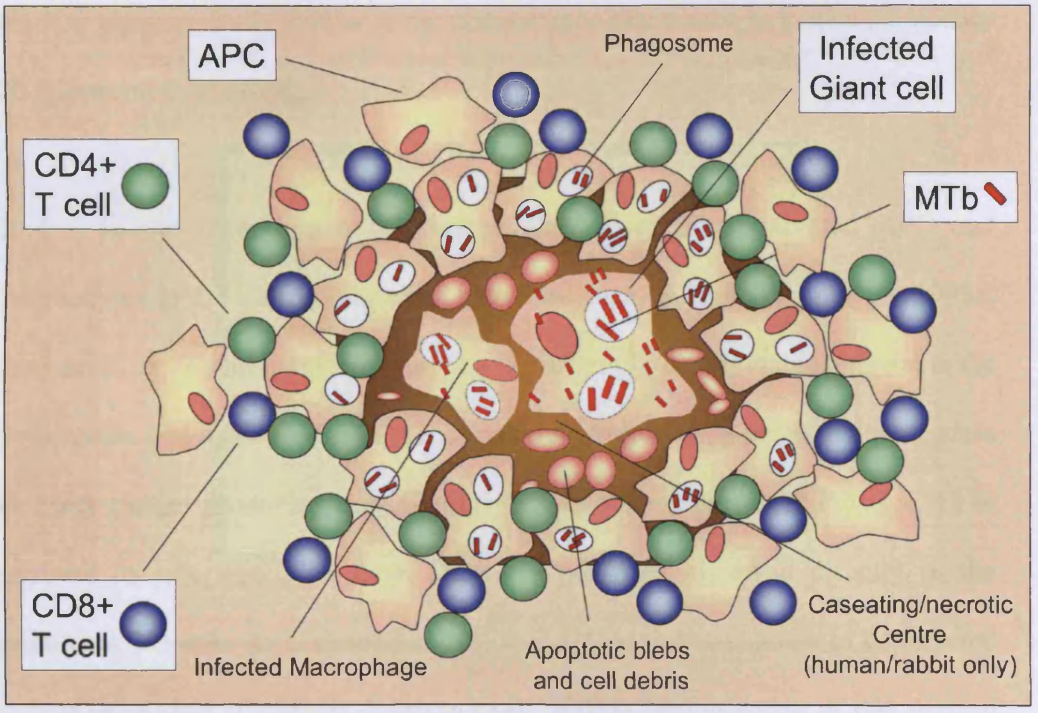


***Figure 1.3*: The caseating tuberculoid granuloma during human MTb infection.**

1.3.3.2. The granulomatous response in animal models of MTb:

The majority of studies on granuloma formation during MTb infection have largely been performed in mice as opposed to guinea pigs and rabbits. This is largely due to factors such as maintenance costs, bio-safety containment space requirements and availability of immunological reagents. The granulomatous response in mice does not progress to caseation or necrosis such as those observed in human pulmonary TB, often giving rise to non-caseating sarcoid granulomas (Fig. 1.4). In addition, this is not to say that the guinea pig and rabbit models are not useful tools in dissecting the immune response to MTb, for example rabbits are the only animal model that following infection both caseation and cavitation are evident (Fig 1.3), which is phenotypically similar to the phenomenon that occurs in human pulmonary TB (Converse *et al.*, 1996).

Studies in the mouse have highlighted the presence of activated APC and lymphocytes in the milieu producing cytokines such as TNF (Flynn *et al.*, 1995a, Bean *et al.*, 1999) and lymphotoxin-alpha (Roach *et al.*, 2001a) that are pivotal in the organisation and maintenance of the granuloma. Surrounding the granuloma centre are macrophages producing pro-inflammatory cytokines such as TNF and IL-12 in response to infection. To date, DC have not been observed directly in the granuloma, however as discussed above, lung DC have been shown to be infected with MTb *in vitro* (Gonzalez-Juarrero *et al.*, 2001a), and infection of BM derived DC gives production of TNF and IL-12 (Bodnar *et al.*, 2001). DC play an essential role in the initiation of adaptive immune responses as following mycobacterial challenge they migrate to draining lymph nodes (Bhatt *et al.*, 2004, Humphreys *et al.*, 2006), where they initiate adaptive immune responses and can give rise to the

generation of antigen specific CD4+ and CD8+ T cells producing IFN-gamma (Feng *et al.*, 1999). Similar to human granuloma studies by Ulrichs et al., (2004), studies in the mouse have reported that CD4+ T cells form organised aggregates and are more abundant within granulomas than CD8+ T cells, which are restricted predominantly to the periphery (Gonzalez-Juarrero *et al.*, 2001b).

The presence of T cell derived IFN-gamma optimally activates macrophages for anti-microbial activity by inducing compounds such as reactive oxygen and nitrogen intermediates (Chan *et al.*, 1992, Shiloh *et al.*, 2000). In response to stress, MTb switches to fatty acid metabolism in order to survive and take advantage of the abundance of lipids and nutrients in the granuloma (McKinney *et al.*, 2000). Furthermore, the cytokine and chemokine signals responsible for controlling the initiation of the granulomatous response in both human and animal models are at present poorly understood (Ulrichs *et al.*, 2006).

The majority of granuloma studies to date have focussed on animal models of MTb. As discussed earlier, the characteristics of granuloma structure and function following MTb infection in animals may differ compared to human hosts. Interestingly, many similarities are evident between human and animal models following MTb infection as following MTb exposure mononuclear cell granulomas develop in the lung followed by strong antigen specific CD4+ and CD8+ T cell responses that control infection. In addition, the infection persists under tight immunological control for many months (North *et al.*, 2004). However, the rabbit model of pulmonary MTb is the only animal model similar to the human with regard to granuloma caseation and cavitation (Converse *et al.*, 1996, McMurray, 2001).

When this phenomenon occurs as a result in a breakdown of protective immunity, vast numbers of infectious bacilli are released in aerosol droplets. Summarised in Table 1.1. are features associated with MTb infection and granuloma assembly in the human as compared to typical animal models: mouse, rabbit and guinea pig.

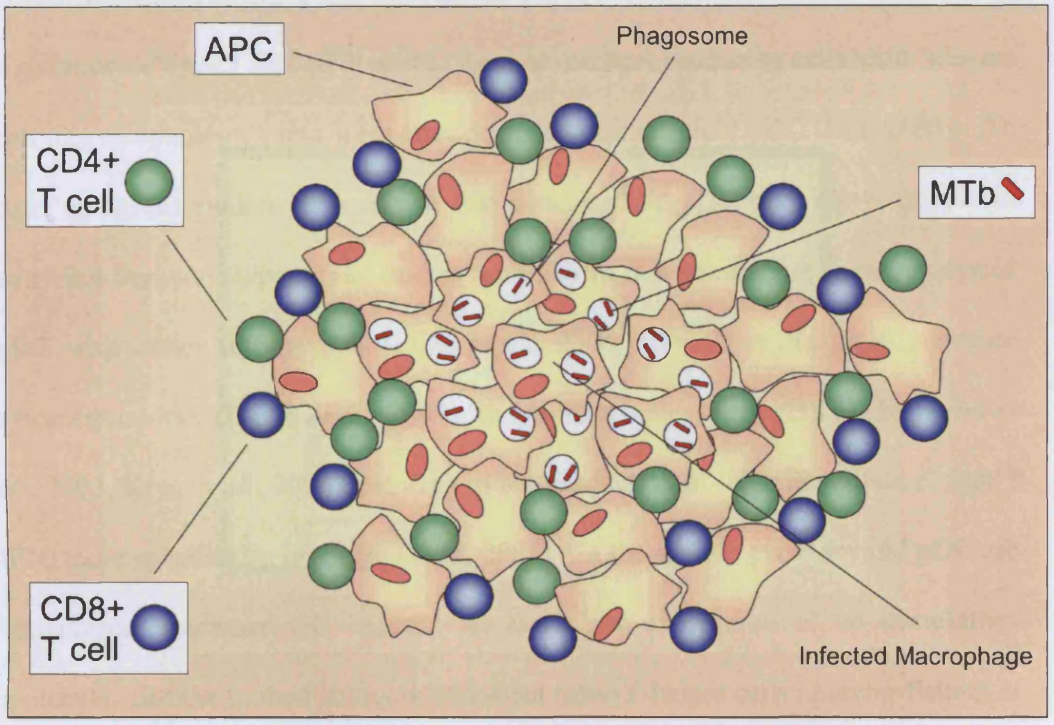


***Figure 1.4*: The non-caseating sarcoid granuloma during murine MTb infection.**

Table 1.1: Comparison of pulmonary MTb infection between the human host and animal models.

Disease feature:	Human:	Mouse:	Rabbit:	Guinea Pig:
Susceptibility to MTb	++	+	+	+++
Extrapulmonary dissemination	++	++	++	++
Hematogenous seeding of apical lobes	++	?	?	++
Typical mononuclear cell granulomas	++	++	++	++
Caseation/necrosis	++	--	++	++
Liquifaction/cavitation	++	--	++	-
Delayed-type hypersensitivity	++	+/-	++	++
Protection by BCG vaccine	++/-	+	++	++
BSL-3 containment space required	NA	+	++++	++
Maintenance costs	NA	+	++++	++
Immunological reagents	++++	++++	++	+

(adapted from McMurray, 2001)

1.3.4. Plasmacytoid pDC:

1.3.4.1. Background:

Studies on human PBMC (Ronnblom *et al.*, 1983), and more recently in the mouse (Asselin-Paturel *et al.*, 2001, Nakano *et al.*, 2001, Bjorck, 2001), described a population of Type I IFN (IFN-alpha, -beta or -omega) producing cells with "plasma cell like morphology", that were later renamed plasmacytoid pDC (Liu, 2005). To date, numerous studies have shown that plasmacytoid pDC play a pivotal role in anti-viral immune response, as studies have shown that stimulation of plasmacytoid pDC with either influenza A (Lund *et al.*, 2004, Diebold *et al.*, 2004), murine cytomegalovirus (Dalod *et al.*, 2003), or herpes simplex virus (HSV) (Izaguirre *et al.*, 2003, Krug *et al.*, 2004), resulted in the production of large quantities of type I IFN, more specifically IFN-alpha (Liu, 2005). In the mouse, plasmacytoid pDC are considered immature DC because of their low expression of co-stimulatory molecules and the limited ability to stimulate naïve T-helper cells (Asselin-Paturel *et al.*, 2001). Because of this observation plasmacytoid pDC are regarded as classic innate cells that produce large amounts of type I IFN e.g. IFN-alpha following viral challenge. However, upon activation plasmacytoid pDC up-regulate co-stimulatory molecules and can present antigen. On the other hand, plasmacytoid pDC have been shown to be regulatory under conditions such as allergy (de Heer *et al.*, 2004) whilst also mediating tolerance to vascularised grafts (Ochando *et al.*, 2006). Furthermore, no studies at present have reported the ability of plasmacytoid pDC to either phagocytose bacteria or play a role during bacterial infection. The role of plasmacytoid pDC during MTb infection will be the focus of chapter 5.

1.3.4.2. Type I IFN: inhibition of the anti-bacterial immune response:

Interestingly, stimulation of mouse but not human plasmacytoid pDC with CpG-DNA drives the production of high levels of IL-12, IFN-alpha but no IL-10 (Boonstra *et al.*, 2006). Therefore the involvement of this DC subset may be important for inducing strong Th1 responses. In line with this, IL-12 is well known to promote protective Th1 responses that are important for clearance of MTb, but its production can be inhibited (Cousens *et al.*, 1997) or enhanced under certain conditions by type I IFN (Gautier *et al.*, 2005). Recent studies have shown enhanced immune responses to *Listeria monocytogenes* in the absence of IFN-alpha/beta signalling (Auerbuch *et al.*, 2004, Carrero *et al.*, 2004, O'Connell *et al.*, 2004). The findings by Auerbuch *et al.*, (2004) indicated that in the absence of the Type I IFN, TNF production by phagocytic cells at the site of infection was enhanced thus promoting bacterial killing. In conclusion, the interplay between IL-12 and type I IFN during infections may therefore be important for the outcome of the immune response to different pathogens.

1.3.4.3. Plasmacytoid pDC, type I IFN and MTb:

As described earlier, infection studies with MTb on macrophages and BM myeloid DC are well documented, although the infection of plasmacytoid pDC with MTb (addressed in chapter 5), has however not been reported. In human studies plasmacytoid pDC have been shown to be present in the lymph nodes of TB patients (Cella *et al.*, 1999) and are decreased in the blood of active TB patients prior to antibiotic chemotherapy (Lichtner *et al.*, 2006). Following successful completion of chemotherapy the numbers of blood DC and plasmacytoid pDC returned to baseline levels (Lichtner *et al.*, 2006). In the mouse model, injection of heat killed MTb into

the footpad gave a 5-10 fold increase in plasmacytoid pDC induction in the knee draining lymph nodes (Blasius *et al.*, 2004). In the same study, *in vitro* stimulation of BM derived plasmacytoid pDC with heat-killed MTb did not induce IFN-alpha (Blasius *et al.*, 2004), but the production of TNF or IL-12 was not examined. In addition, it has been suggested that the hyper-virulent phenotype of the clinical isolate MTb HN878 in mice is due to induction of type I IFN, however, the source of the Type I IFN was not examined (Manca *et al.*, 2001, Manca *et al.*, 2005). Therefore, the role of plasmacytoid pDC as a source for Type I IFN during MTb infection is still at present unclear.

In summary, the involvement of plasmacytoid pDC in regulating the immune response to invading pathogens can be mediated, a) directly through production of large quantities IFN-alpha/beta in response to viruses, or b) indirectly via IFN-alpha/beta regulation of IL-12 as highlighted above. Similarly, plasmacytoid pDC may also regulate asthmatic responses to inhaled antigens (de Heer *et al.*, 2004) or tolerance to grafts (Orchando *et al.*, 2006) through unknown mechanisms.

1.3.5. The importance of the Th1 response in immunity to intracellular pathogens:

IL-12 is essential for driving CD4+ T cells towards a Th1 response (characterised by the production of IFN-gamma), which is key mediating immunity to intracellular pathogens such as MTb. However, IL-4 production promotes the Th2 response (characterised by the production of IL-4, IL-5 and IL-13), which is responsible for mediating protection to extracellular pathogens such as helminths, but in addition

may also promote allergic reactions. IL-17 producing cells (Th-17) are a newly described subset of highly inflammatory CD4+ T cells that have been implicated in autoimmune tissue injury leading to diseases such as experimental allergic encephalomyelitis (EAE) (Park *et al.*, 2005, Langrish *et al.*, 2005). Th-17 cells are induced following microbial stimulus whilst in the presence of TGF-beta and IL-6 (Mangan *et al.*, 2006, Veldhoen *et al.*, 2006) and their expansion requires the IL-12 family cytokine IL-23 (Harrington *et al.*, 2005).

1.3.5.1. The Th1 response in human MTb: lessons learnt from Mendelian susceptibility cases.

The essential requirement for a Th1 response for control of mycobacterial infections has been observed in patients exhibiting Mendelian susceptibility to mycobacterial diseases. Mutations in the IL-12 receptor or related signalling molecules render individuals extremely susceptible to intracellular pathogens such as atypical mycobacteria (Altare *et al.*, 1998, Casanova *et al.*, 2002, Dorman *et al.*, 2000), *Salmonella* (de Jong *et al.*, 1998) and pulmonary TB (Remus *et al.*, 2004). Similarly, mutations in the IFN-gamma receptor 1 also render individuals extremely susceptible to pulmonary TB (Newport *et al.*, 2003), BCG (Jouanguy *et al.*, 1996) and environmental mycobacteria (Dorman *et al.*, 2004). Interestingly, it has also shown that individuals with a polymorphism in the promoter for monocyte chemo-attractant protein-1 (MCP-1) have enhanced levels of MCP-1 that inhibited the production of IL-12p40 by monocytes in response to MTb stimulation (Flores-Villanueva *et al.*, 2005).

1.3.5.2. The Th1 response in mouse MTb:

1.3.5.2.1. Cytokines:

The findings in human TB patients highlighting the importance of IFN-gamma and IL-12 have also been demonstrated in the mouse. Knockout mice deficient in IFN-gamma (Flynn *et al.*, 1993, Cooper *et al.*, 1993) or IL-12 (Flynn *et al.*, 1995b, Cooper *et al.*, 1995, Cooper *et al.*, 1997, Cooper *et al.*, 2002) succumb early to MTb infection with high bacterial loads (Fig. 1.5).

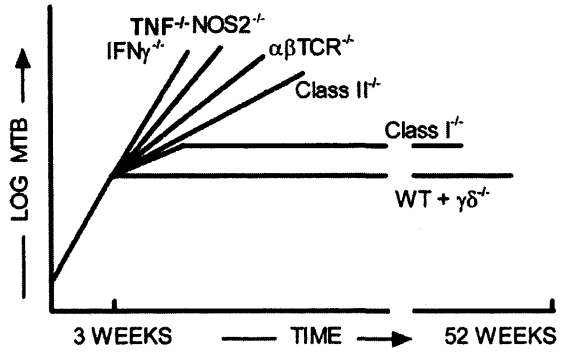


***Figure 1.5**: A simplified overview of the experimental studies in the mouse highlighting the importance of CD4+ T cells, CD8 T cells, IFN-gamma and TNF in the anti-MTb response.* **(adapted from North *et al.*, 2004).**

The importance of a strong Th1 response mediated by IFN-gamma following MTb infection has been well documented, interestingly, studies have also shown that as MTb infection progresses to chronicity a Th2 phenotype where the production of IL-4 becomes apparent (Orme *et al.*, 1993, Hernandez-Pando *et al.*, 1996), which also correlates with disease reactivation (Howard *et al.*, 1999). Studies in mice using adoptive transfer of Th1 and Th2 cells (polarised *in vitro*) at the time of infection have shown that although Th1 cells mediate protection during infection, transfer of Th2 cells was associated with enhanced lung pathology and weight loss (Wangoo *et*

al., 2001). These findings suggest that the switch to a Th2 phenotype during chronic *in vivo* infection may function to dampen the immune response and limit host pathology.

1.3.5.2.2. The protective role of different T cell subsets in the immune response to MTb:

The essential role of CD4+ and to a lesser extent CD8+ T cells has also been well documented during MTb infection. Mice deficient in CD4+ T cells (major histo-compatibility complex [MHC]-$II^{-/-}$) (Caruso *et al.*, 1999) or mice depleted of CD4+ T cells via antibody depletion (Scanga *et al.*, 2000) are unable to control aerosol challenge and rapidly succumb to infection. However, in mice lacking CD8+ T cells ($Beta_2$-microglobulin$^{-/-}$ or MHC-$I^{-/-}$) (Flynn *et al.*, 1992, Sousa *et al.*, 2000, Rolph *et al.*, 2001), or mice depleted of CD8+ T cells via antibody depletion (Mogues *et al.*, 2001), still exhibit protection against MTb challenge but the infection is sustained at a higher level (1-Log_{10}) compared to wild-type mice (Fig. 1.5). Various hypotheses have been put forward identifying differing roles of CD4+ and CD8+ T cell subsets during infection. Reports have suggested that CD4+ T cells play a dominant role as IFN-gamma producers during early infection, and CD8+ T cells serve as an essential source of IFN-gamma and cytolytic granules e.g. perforin, during latter stages of chronic infection (van Pinxteren *et al.*, 2000, Lazarevic *et al.*, 2005). In contrast, studies by Feng *et al.*, (1999) have observed an equal role for both CD4+ and CD8+ T cells during early infection. These findings perhaps reflect the different strains of MTb used between the studies. Reports on the role of gamma/delta T cells using knockout mice has thus far failed to identify a protective role for this T cell subset with regard to regulation of bacterial load. However, mice deficient in this T cell

subset have enhanced lung immune-pathology following aerosol infection compared to wild-type controls (D'Souza *et al.*, 1997) and recent findings by Lockhart *et al.*, (2006) have shown gamma/delta T cells as major producers of IL-17 during early MTb infection.

1.3.5.2.3. IL-23, IL-17 and mycobacterial immunity:

More recently, it has been shown that the IL-12 family cytokine IL-23, can compensate in the absence of IL-12p70 and function during MTb infection as a protective cytokine (Khader *et al.*, 2005, Wozniak *et al.*, 2006). In addition IL-23 is also essential for the expansion of pro-inflammatory Th-17 CD4+ T cells (Harrington *et al.*, 2006). IL-17 is a major inducer of neutrophils during an inflammatory response, in line with this, Th-17 cells have been shown to play a role in autoimmune diseases such as EAE (Park *et al.*, 2005, Langrish *et al.*, 2005). In mycobacterial studies, IFN-gamma production regulates the IL-17 response during infection with BCG (Cruz *et al.*, 2006, Khader *et al.*, 2005), and during MTb infection, gamma/delta T cells are suggested to be the predominant producers of this cytokine (Lockhart *et al.*, 2006). The exact role of this novel IL-17 producing Th subset in mediating either protection or potentially pathology during MTb infection is at present not fully understood.

1.3.5.3. The importance of TNF in controlling MTb reactivation:

The role of TNF during murine MTb infection has to date been well documented. Mice deficient in TNF succumb to MTb around day 30 post infection with enhanced bacterial levels, reduced production of reactive nitrogen intermediates and

disorganised granulomas (Flynn *et al.*, 1995a, Bean *et al.*, 1999). Similarly, TNF is important in mediating bacterial control during latent infection, as treatment of MTb infected-antibiotic cured mice with anti-TNF antibody resulted in disease reactivation (Scanga *et al.*, 1999, Mohan *et al.*, 2001).

Similar to the findings in mice, recent studies by Keane *et al.*, (2001) during human MTb infection, have also highlighted the importance of TNF. They reported that latent (non-active) TB patients receiving anti-TNF therapy (Infliximab) for rheumatoid arthritis (RA) or Crohn's disease may develop active TB soon after initiation of Infliximab treatment. The baseline level of TB in RA patients in the USA is approximately 6 per 100,000, the incidence of TB rises to 24 per 100,000 in RA patients receiving Infliximab therapy (Keane *et al.*, 2001).

1.3.6. The role of CD1 molecules in MTb lipid presentation:

1.3.6.1. CD1 expression in humans:

The role of CD1 molecules is to regulate immunity not only to self, in order to prevent autoimmunity, but also to initiate anti-bacterial responses upon encountering microbial lipids (Brigl *et al.*, 2004). In the human, CD1 molecules can be subdivided into Group 1 (CD1a, CD1b and CD1c - expressed exclusively on DC and APC), Group 2 (CD1d - expressed on DC and APC but in addition on epithelial cells parenchymal cells and vascular smooth muscle cells in the liver and gut) and intermediate molecules (CD1e). Following activation, CD1 restricted T cells can mediate helper, effector and adjuvant like functions by interacting with DC, macrophages, NK cells, T cells and B cells (Brigl *et al.*, 2004).

1.3.6.2. CD1 expression in mice:

In contrast, mice only possess CD1d and present antigen to invariant Natural Killer (iNK) T cells (De Libero *et al.*, 2005). Stimulation of iNKT cells with lipid antigen can lead to the production of the cytokines IFN-gamma, IL-4, IL-13, TNF and GM-CSF along with cytolytic granules, thereby demonstrating an important role in linking the innate and adaptive immune response (De Libero *et al.*, 2005).

1.3.6.3. Characterisation of CD1 restricted antigens:

To date, reports characterising lipid antigens have focused on cell wall constituents from MTb including phosphatidylinositol mannosidase (PIM), lipoarabinomannan (LAM) and didehydroxymycobactin (DHM) (Moody *et al.*, 2004). Both MTb derived PIM, LAM and DHM can be bound by CD1 molecules that are expressed predominantly but not exclusively on DC, macrophages and B cells in order to activate appropriate T cell responses (Winau *et al.*, 2004a). In addition, studies have identified mycobacterial cell wall constituents that are presented on specific CD1 molecules: CD1a = DHM; CD1b = PIM and LAM; CD1c = Mannosyl-beta-1-phosphomycoketide; CD1d = phosphatidyl-tetra-mannoside (De Libero *et al.*, 2005).

1.3.6.4. The role of CD1 in the alternative "detour" pathway during MTb infection:

Recent findings by Schaible *et al.*, (2003), have identified a "detour" pathway for activation of CD8+ T cells via recognition of antigen by MHC-I and CD1. This significant finding demonstrated that during MTb infection, macrophages release apoptotic vesicles containing MTb antigens that can "cross prime" T cells via un-infected bystander DC. Bystander DC uptake the vesicles containing antigen,

process and present them via MHC-I and CD1 in order to facilitate the activation of CD8+ T cells (Schaible *et al.*, 2003). During cross priming by DC, the chaperone type molecules called saposins, have been shown to be essential in the loading of MTb lipids onto CD1b molecules in human APC (Winau *et al.*, 2004b).

1.3.6.5. Exploiting CD1 presentation as a strategy during BCG vaccination or cancer immunotherapy to boost sub-optimal immune responses:

Grode *et al.*, 2005 have highlighted the potential for exploiting CD8+ T cell responses mediated through CD1 lipid presentation, in order to enhance the efficacy of the current BCG vaccine. The recombinant BCG (rBCG) used expressed the membrane perforating factor listeriolysin (derived from *Listeria monocytogenes*). Upon phagosomal acidification in the APC, the listeriolysin would become activated and facilitate rBCG escape into the cytosol leading to cross priming and initiation of CD1 and MHC-I presentation to CD8+ T cells (Grode *et al.*, 2005). The benefits of targeting CD1 restricted T cells is not just restricted to MTb or mycobacterial diseases. It has been reported using a murine model of cancer that targeting CD1d restricted NK T cells via delivery of alpha-galactosylceramide in conjunction with pre-existing therapy led to eradication of established tumours (Silk *et al.*, 2004).

1.4. The role of TLR in initiating anti-microbial responses:

1.4.1. Overview:

TLR are a diverse family of innate immune recognition receptors that are expressed on B cells, macrophages, DC and neutrophils. TLR recognise pathogen associated molecular proteins (PAMP) and induce antimicrobial immune responses (Medzhitov,

2001, Akira *et al.*, 2004). PAMP bind TLR expressed on the surface of immune cells, in doing so adaptor molecules such as MyD88, TIRAP, TRIF and TRAM are activated and recruited resulting in a signalling cascade, cell activation and cytokine production (Fig. 1.6).

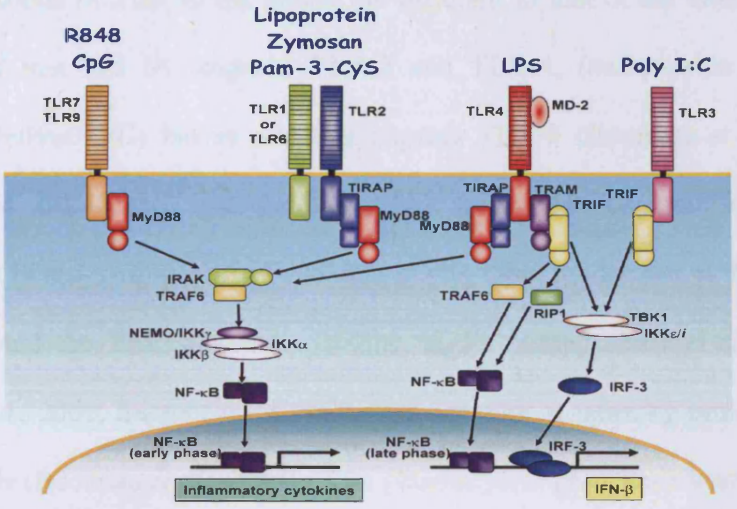


Figure 1.6: ***TLR ligands and their specific intracellular signalling adaptor molecules.*** **(Adapted from Takeda *et al.*, 2005). TIRAP – Toll IL-1R associated protein, TRIF – TIR domain containing adaptor protein inducing Interferon Beta, TRAM – TRIF related adaptor molecule.**

1.4.2. TLR expression in humans and mice:

There are 11 known TLR humans and mice. TLR expression has been shown to be cell specific, in humans it has been reported by (Kadowaki *et al.*, 2001) that monocytes express TLR-1, 2, 4, 5 and 8, CD11c+ immature blood DC express TLR-3 and plasmacytoid pDC exclusively express TLR-7 and TLR-9. However, the distribution of human TLR has been shown to vary and the criteria are not as rigid as Kadowaki *et al.*, (2001) portrays. For example, studies by Muzio *et al.*, (2000) have shown that monocyte derived DC express TLR-3 and is therefore not exclusively

expressed on CD11c+ immature DC. Furthermore, it is possible that TLR may be regulated on macrophages, DC, neutrophils and B cells during an immune response as recently shown for human leukocytes (Muzio *et al.*, 2000).

The distributions of TLR in the mouse are different to that of the human. In the mouse, BM myeloid DC express TLR-2 and TLR-4, (comparable to human monocyte-derived DC) but in addition express TLR-9 (Boonstra *et al.*, 2003). Plasmacytoid pDC derived from the BM or their splenic derived equivalent are well documented in responding to viral infection giving rapid production of the cytokine IFN-alpha and the Th1 inducing cytokine IL-12 (Asselin-Paturel *et al.*, 2001, Nakano *et al.*, 2001, Barchet *et al.*, 2002), but are poor at inducing proliferation of naïve T cells (Boonstra *et al.*, 2003). The plasmacytoid pDC have been shown by Boonstra *et al.*, (2003) to express largely TLR-9 but also TLR-7, therefore responding strongly to the TLR-9 ligand CpG-DNA and TLR-7 ligand R848 (Imadizolquinolene) in keeping with findings in human studies. In murine studies CpG-DNA is a potent inducer of pro-inflammatory cytokines including IL-12 from macrophages, DC and plasmacytoid pDC. CpG is non-methylated (unlike human DNA) and is present in MTb as well as viruses and other organisms. The expression of TLR-9 on mouse BM myeloid DC and splenic macrophages is distinct from human since in human TLR-9 is exclusively expressed on plasmacytoid pDC and B cells but has been recently suggested to be up-regulated by MTb products (Muzio *et al.*, 2000).

1.4.3. TLR in MTb:

Initial studies addressing the role of TLR during MTb infection have highlighted controversial roles for single TLR in mediating protective immune responses. The involvement of TLR-2 and TLR-4 in mediating TNF responses during *in vitro* MTb infection have been reported using human TLR-transfected cell lines (Means *et al.*, 1999, Means *et al.*, 2001). Similar findings have also been reported in the mouse by Underhill *et al.*, (1999), where TNF production by murine macrophages in response to MTb was dependent on TLR-2 and MyD88. *In vivo* studies in TLR-$4^{-/-}$ mice have reported a role for this TLR in mediating protection from aerosol challenge (Abel *et al.*, 2002). However, there is evidence to suggest that TLR-2, but not TLR-4 is responsible for mediating protection from aerosol MTb challenge (Reiling *et al.*, 2002). These findings suggest that other factors may be involved. More recently, studies by Bafica *et al.*, (2005) have demonstrated a role for TLR-9 during aerosol MTb challenge. Following MTb infection, TLR-$9^{-/-}$ mice had comparable bacterial levels to control mice but IL-12p40 and CD4+ T cell derived IFN-gamma were significantly decreased. In the same study it was also demonstrated that multiple TLR can collaborate in order to regulate immunity to MTb challenge as TLR2/9 double knockout mice had elevated bacterial loads compared to single knockout controls (Bafica *et al.*, 2005).

1.5. Regulatory mechanisms involved in the down regulation of the immune response to infection: studies from human and mouse:

1.5.1. The role of IL-10 in regulating immunity to invading pathogens:

1.5.1.1. The biology of IL-10:

IL-10 is an immunosuppressive cytokine displaying anti-inflammatory functions. IL-10 is produced by a variety of cell types including B cells, T cells, macrophages and some DC (Moore *et al.*, 2001). IL-10 can inhibit the production of pro-inflammatory cytokines such as TNF and the Th1 polarising cytokine IL-12 (Fig. 1.7) by directly acting on DC and macrophages (Fiorentino *et al.*, 1991a, Fiorentino *et al.*, 1991b) to inhibit pathogen killing (Gazzinelli *et al.*, 1992, Moore *et al.*, 2001).

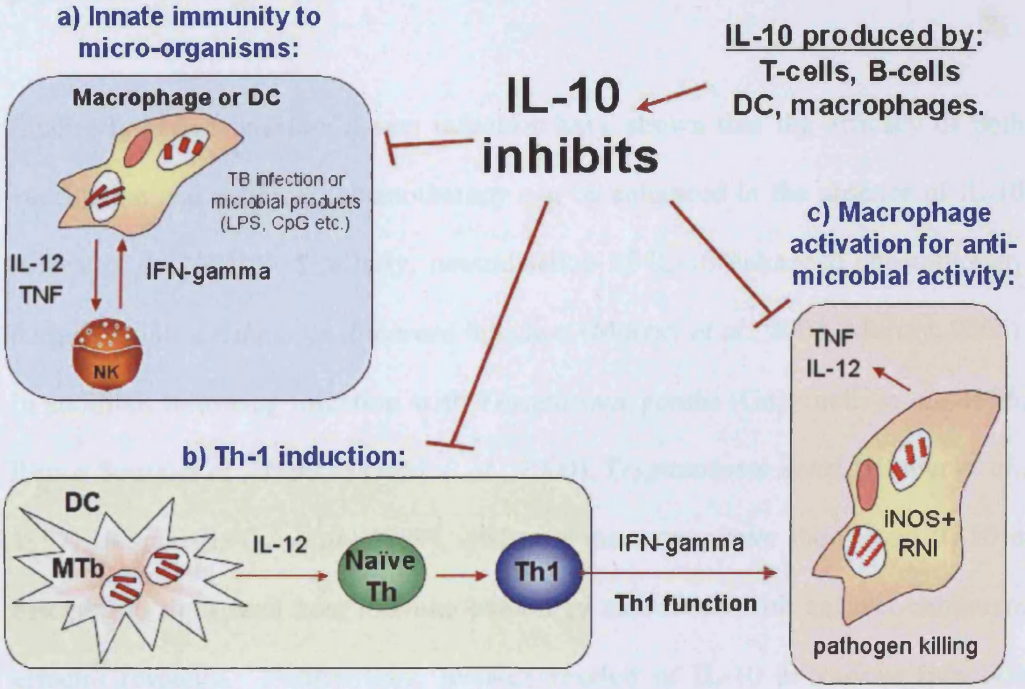


***Figure 1.7**: The source and immunosuppressive role of IL-10 in regulating protective immunity to infection.*

1.5.1.2. IL-10 in infectious disease:

When addressing the role of IL-10 in the mouse, studies using *Listeria monocytogenes* (Wagner *et al.*, 1994, Dai, 1997, Silva *et al.*, 2001a), *Leishmania major* (Belkaid *et al.*, 2001, Belkaid *et al.*, 2002), *Leishmania donovani* (Murray *et al.*, 2002), *Mycobacterium avium* (Bermudez *et al.*, 1993, Roach *et al.*, 2001b) and BCG (Murray *et al.*, 1999), have all reported an increase in pathogen clearance in the absence of IL-10. Strikingly, in *Leishmania major*, although the absence of IL-10 led to enhanced pathogen clearance, mice displayed a loss of immunity to re-infection (Belkaid *et al.*, 2002). This finding suggests that IL-10 does limit pathogen clearance, but moreover plays a key role in the maintenance of effector memory populations although at present the mechanism is unknown.

Studies in *Mycobacterium avium* infection have shown that the efficacy of both vaccination and antibiotic chemotherapy can be enhanced in the absence of IL-10 (Silva *et al.*, 2001b). Similarly, neutralisation of IL-10 enhanced chemotherapy during chronic *Leishmania donovani* infection (Murray *et al.*, 2002, Murray, 2005). In addition, following infection with *Toxoplasma gondii* (Gazzinelli *et al.*, 1996, Reis e Sousa *et al.*, 1999, Suzuki *et al.*, 2000), *Trypanosoma cruzi* (Hunter *et al.*, 1997) and malaria (Li *et al.*, 1999), studies in the mouse have shown that IL-10 is essential to limit fatal host immune-pathology associated with an over-exuberant effector response. Furthermore, over-expression of IL-10 in various infection systems has also shown a regulatory role for this cytokine, as expression of IL-10 by a recombinant strain of *Mycobacterium smegmatis* can inhibit activation and pro-inflammatory cytokine production by macrophages *in vitro* (Marshall *et al.*, 1997). Similarly, studies in *Mycobacterium avium* using a transgenic mouse that over-

expresses IL-10, have shown that infected mice have enhanced bacterial levels and impaired macrophage function (Feng *et al.*, 2002). These findings above highlight the regulatory role that IL-10 plays in limiting protective effector responses but at the same time inadvertently hampering pathogen eradication. The potential regulatory role for IL-10 during MTb infection will be addressed in chapter 3.

1.5.1.3. IL-10 and MTb:

1.5.1.3.1. The role of IL-10 in human MTb studies:

Studies on PBMC obtained from infected TB patients, have shown that neutralisation of endogenous IL-10 increased the production of IFN-gamma and IL-12p40 in response to heat-killed MTb (Gong *et al.*, 1996). Furthermore, studies on PBMC obtained from in anergic TB patients i.e. TB patients that have no positive purified protein derivative (PPD) skin test reaction, have demonstrated that neutralisation of endogenous IL-10 enhances proliferative responses to PPD (Boussiotis *et al.*, 2000). The studies by Gong *et al.*, (1996) and Boussiotis *et al.*, (2000), concluded that IL-10 was functioning to limit the immune response to MTb. In addition, Gerosa *et al.*, (1999) have reported the presence of CD4+ T cells producing both IFN-gamma and IL-10 in the broncho-alveolar lavage (BAL) of active TB patients. These findings in the BAL were in contrast to CD4+ T cells derived from the peripheral blood that were of a Th2 phenotype producing both IL-10 and IL-4 (Gerosa *et al.*, 1999). The suppressive action of endogenous IL-10 and TGF-beta has been shown to inhibit CD4+ T cell proliferation and IFN-gamma production in MTb stimulated PBMC obtained from healthy PPD+ patients although there was no effect on MTb uptake by monocytes (Rojas *et al.*, 1999). In

conclusion, the human studies of TB infection suggest that during chronic MTb endogenous IL-10 may inhibit protective Th1 responses via indirect action on macrophages or DC and may ultimately be functioning to limit immune-pathology. However, these studies failed to address the relationship between IL-10 and its suppressive effect on pathogen eradication.

1.5.1.3.2. The role of IL-10 in murine MTb studies:

The literature to date when addressing the role of IL-10 during murine MTb infection is controversial with studies focusing predominantly on IL-10 deficient (*Il10*$^{-/-}$) mice. Studies have reported that *Il10*$^{-/-}$ mice are no more resistant or susceptible to aerosol MTb infection than wild type mice (North, 1998, Jung *et al.*, 2003). In contrast, others have reported that *Il10*$^{-/-}$ mice display a transient increase in resistance to aerosol MTb challenge in the first 4 weeks following infection, but by week 8 and 12, they were no more resistant to MTb than wild-type mice (Roach *et al.*, 2001b).

Thus far, the literature suggests that endogenous IL-10 has little to no effect during the early phase of MTb infection. However during late MTb infection of IL-10 transgenic mice, where IL-10 is under the control of the IL-2 promoter, bacterial burdens in transgenic mice are elevated compared to wild type mice, thus confirming the suppressive effect of IL-10 on the anti-MTb response. However, this model of MTb infection by Turner *et al.*, (2002) is not physiological, in that not all IL-2 producing cells in the mouse would actually produce IL-10 and secondly the IL-10 production is not under the natural control of its own promoter. We will address the

potential regulatory role for IL-10 in murine studies of both early and late MTb infection.

1.5.2. TGF-beta and immune-regulation:

1.5.2.1. Overview:

TGF-beta is an immunosuppressive cytokine produced in three distinct isoforms; TGF-beta 1, 2, and 3. TGF-beta 1 is the most dominant form in lymphoid organs being produced by all leucocytes, exerting an inhibitory effect on Th1 and Th2 responses, macrophage activation and DC maturation (Letterio *et al.*, 1997, Li *et al.*, 2006a). In addition, TGF-beta also plays an essential role in many developmental and physiological processes, as *Tgf-B1*$^{-/-}$ mice die at 3-4 weeks of age due to an overwhelming lymphoproliferative disorder (Shull *et al.*, 1992). TGF-beta can indirectly inhibit the development of a protective Th1 response e.g. following *Leishmania* infection, by inhibiting IFN-gamma production from NK cells (Laouar *et al.*, 2005). In line with this, TGF-beta has also been shown to decrease the survival of BCG activated T cells (Hernandez-Garay *et al.*, 2003). Similarly, regulatory T cells (Tregs) producing TGF-beta can modulate the function of effector cells during colitis (Powrie *et al.*, 1996, Fahlen *et al.*, 2005). It has also been reported that T cell receptor stimulation in the presence of TGF-beta has been implicated in the conversion of peripheral naïve CD4+CD25- T cells into CD4+CD25+ Tregs (Chen *et al.*, 2003, Fantini *et al.*, 2004) that can be inhibited in the presence of IL-6 (Bettelli *et al.*, 2006). TGF-beta is also an essential factor involved in the maintenance of the Treg population (Marie *et al.*, 2005).

1.5.2.2. The role of TGF-beta during MTb infection:

The role of TGF-beta during MTb infection has not been well documented and is addressed in chapter 4 where we examined its potential for down regulating protective immune responses to MTb. In human studies it has been found that TGF-beta is increased in the serum of TB patients (Fiorenza *et al.*, 2005, Guyot-Revol *et al.*, 2006) and can inhibit CD4+ T cell proliferation and IFN-gamma production by PBMC (Rojas *et al.*, 1999).

In rodent studies, TGF-beta expression has been shown to increase in the lungs of aerosol infected mice (Mogga *et al.*, 2003). TGF-beta can also be induced *in vitro* following stimulation of MTb secretory antigen matured DC with MTb cell extract (Balkhi *et al.*, 2004). Neutralisation of TGF-beta in guinea pigs injected with heat-killed MTb gave enhanced TNF and reduced cellular infiltrate into the pleural space (Allen *et al.*, 2004). However, IFN-gamma responses were unaffected following anti-TGF-beta neutralisation.

1.5.3. Regulatory T cells (Tregs) as negative regulators of the immune response to invading pathogens:

1.5.3.1. The biology of Tregs:

Tregs mediate suppressive activity either via cell-cell contact or through production of suppressive cytokines e.g. IL-10 or TGF-beta. The outcome limits aberrant autoimmune responses or may dampen over-exuberant and potentially pathological innate or adaptive immune responses to antigen or infection. The presence of regulatory factors may benefit the host by limiting collateral damage but may

inadvertently promote pathogen persistence. Tregs have been subdivided into a) naturally occurring Tregs derived from the thymus or b) antigen driven IL-10 producing CD4+ Tregs (IL-10 Tregs) that result after exposure to specific stimulatory conditions during infection or antigen exposure (Mills, 2004, O'Garra *et al.*, 2004, Belkaid *et al.*, 2005). The role of Tregs and Treg derived IL-10 as negative regulators of the immune response to MTb will be addressed in chapter 4.

1.5.3.2. Naturally occurring Tregs:

1.5.3.2.1. Overview:

Naturally occurring CD4+CD25+ Tregs were originally described in the mouse (Sakaguchi *et al.*, 1995) and more recently in humans (Levings *et al.*, 2001), as essential regulators for autoimmunity, allergy and disease (Hawrylowicz *et al.*, 2005). Initial studies demonstrated that depletion of CD25+ cells from whole CD4+ T cells and then subsequently transferred into lymphopenic hosts resulted a broad range of autoimmune disorders including thyroiditis and gastritis (Sakaguchi *et al.*, 1995). In adult mice and humans CD4+CD25+ Tregs constitute approximately 5-10% of peripheral CD4+ T cells and 1% of CD8 T cells (Fontenot *et al.*, 2005a). Tregs can be isolated from humans and naïve mice via the marker CD25 (IL-2 receptor [IL-2R]-alpha), which is however also expressed on activated T cells (Shevach, 2006). Furthermore, recent phenotypic analysis of murine Tregs has shown that in addition to original markers CD4 and CD25, Tregs also express the markers CTLA-4, glucocorticoid TNF receptor (GITR), CD103 (alpha-$_E$ integrin) and OX40 (Fontenot *et al.*, 2005b). Similar to CD25, these molecules are also expressed on activated T cells, which makes functional Treg depletion studies i.e. by

anti-CD25 treatment of mice, difficult to interpret (Sakaguchi *et al.*, 2006, Shevach, 2006). With this observation in mind, alternative approaches such as adoptive Treg cell transfer have been utilised. More recently, naturally occurring Tregs were shown to express the forkhead (winged helix) transcription factor forkhead box P3 (FoxP3) (Fontenot *et al.*, 2003, Hori *et al.*, 2003, Khattri *et al.*, 2003), which is fundamental for their development and function.

1.5.3.2.2. Treg mediated suppression of auto-immunity:

Studies by Powrie and colleagues identified the presence of Tregs contained in a population of CD4+CD45RB^{low} T cells. This population of CD4+CD45RB^{low} T cells have the ability to regulate Th1 mediated colitis *in vivo* by CD4+CD45RB^{high} T cells through TGF-beta (Powrie *et al.*, 1996, Fahlen *et al.*, 2005), IL-10 (Asseman, 1999, Asseman *et al.*, 2003) and CTLA-4 dependent mechanisms (Read *et al.*, 2000, Read *et al.*, 2006). Furthermore, CD4+CD25+ Tregs can inhibit Th1 mediated colitis in SCID mice in an antigen specific manner when co-infected with the parasite *Leishmania major* (Xu *et al.*, 2003), and is dependent on IL-10, TGF-beta and CTLA-4 (Liu *et al.*, 2003). In contrast to these observations *in vivo*, *in vitro* Treg suppression assays by have shown that regulation is mediated by a cell-cell contact dependent mechanism and not via IL-10, TGF-beta or CTLA-4 (Thornton *et al.*, 1998, Vieira *et al.*, 2004). Therefore the mechanism of suppression utilised by Tregs is at present poorly understood.

1.5.3.2.3. Characterisation of the Treg specific transcription factor FoxP3:

As discussed earlier, studies by Fontenot *et al.*, (2003), Hori *et al.*, (2003) and Khattri *et al.*, (2003) identified FoxP3 a transcription factor specific to Tregs. Similar to IPEX (immune dysregulation, polyendocrinopathy enteropathy, X linked) syndrome in humans, scurfin or FoxP3 deficiency in mice results in a lack of Tregs leading to the development of a fatal lymphoproliferative syndrome 3-4 weeks after birth. Expression of FoxP3 is restricted predominantly to CD4+CD25+ Tregs that possess regulatory properties *in vitro* (Fontenot *et al.*, 2003, Hori *et al.*, 2003, Khattri *et al.*, 2003) and *in vivo* (Fontenot *et al.*, 2003). In addition, Khattri *et al.*, (2003) also demonstrated that forced expression of FoxP3 in *Ctla4*$^{-/-}$ mice rescued them from a fatal lymphoproliferative disorder. Interestingly, ectopic expression of FoxP3 in naïve CD4+CD25- T cells, generates a CD4+CD25- T cell with regulatory phenotype that inhibits wasting disease and inflammatory bowel disease in lymphopenic hosts (Fontenot *et al.*, 2003, Hori *et al.*, 2003, Khattri *et al.*, 2003). Additionally, FoxP3 was also required for the development and function of Tregs (Fontenot *et al.*, 2003). In summary, FoxP3 is a unique cell lineage specific transcriptional factor responsible for the development of Tregs and as a marker of naturally occurring Tregs

1.5.3.3. Antigen driven IL-10 Tregs:

As discussed earlier, Tregs can be derived from the thymus as naturally occurring Tregs or induced under specific stimulatory conditions to become IL-10 Tregs (Groux *et al.*, 1997) demonstrated that repeated stimulation *in vitro* of murine DO11.10 (OVA specific) transgenic T cells with OVA peptide in the presence of IL-

10 could drive a distinct population of IL-10 producing Tregs. The resulting Treg cells produced IL-10, little IL-2 and no IL-4 with the ability to prevent colitis in SCID mice re-constituted with splenic CD4+CD45RB^{high} T cells.

Further studies in the mouse have reported that IL-10 producing Tregs can also be obtained following *in vitro* culture with the immunosuppressive drugs vitamin-D3 and dexamethasone, the resulting homogenous IL-10 Treg population can inhibit EAE *in vivo* (Barrat *et al.*, 2002). These *in vitro* driven IL-10 Tregs share similar properties to naturally occurring Tregs in that they can inhibit naïve CD4+ T cell proliferation by cell-cell contact that was independent of IL-10 (Vieira *et al.*, 2004). However, these IL-10 Tregs did not express the transcription factor FoxP3. Interestingly, unlike freshly isolated naturally occurring Tregs, IL-10 Tregs readily produced IL-10 without re-stimulation, suggesting that the importance of "seeing" antigen is key both *in vivo* or *in vitro* in order to drive IL-10 dependent suppression. Furthermore, these findings highlight the different developmental origins of Treg subsets.

Similarly, *in vivo* studies have shown that IL-10 Tregs can be obtained from mice anergised with soluble antigens such as myelin basic protein (Burkhart, 1999, Sundstedt *et al.*, 2003) or following infection with virus (Buer, 1998) or viral super-antigens (Papiernik *et al.*, 1997). In these *in vivo* scenarios, antigen specific CD4+ T cells develop that produce IL-10, little IL-2, no IFN-gamma or IL-4.

1.5.3.4. Tregs in microbial infections:

1.5.3.4.1. Overview:

The roles of Tregs in diseases such as *Helicobacter hepaticus* (Maloy *et al.*, 2003), *Listeria monocytogenes* (Kursar *et al.*, 2002), *Plasmodium* (Hisaeda *et al.*, 2004), *Schistosoma mansoni* (McKee *et al.*, 2004), HIV (Andersson *et al.*, 2005) and HSV (Suvas *et al.*, 2003) have been documented. The role of Tregs during parasitic infection, most notably in murine models of *Leishmania*, has been extensively studied to date. The insight acquired from these *Leishmania* studies has aided and facilitated our understanding of Tregs in immune-regulation, thus enabling us to transfer this knowledge to other infectious diseases.

1.5.3.4.2. Tregs in Leishmania:

1.5.3.4.2.1. Human Leishmania studies:

In human studies of cutaneous *L. viannia braziliensis* infection, Tregs have been associated with promoting parasite persistence as reports have shown the presence of CD4+CD25+FoxP3+ Tregs producing large quantities of IL-10 and TGF-beta (Campanelli *et al.*, 2006). In addition, these Tregs could also inhibit proliferative T cell responses *in vitro*. In contrast, other sources of IL-10 during *Leishmania* infection have been reported. In *Leishmania donovani*, splenic CD4+CD25-FoxP3- T cells can be isolated from patients and are the predominant producers of IL-10 (Sacks, 2006).

1.5.3.4.2.2. Murine Leishmania studies:

As discussed previously, Belkaid *et al.*, (2001) identified IL-10 as a regulator of the protective Th1 immune response in clearance of *L. major* infection in mice. Further analysis using an adoptive transfer model into $Rag^{-/-}$ mice, demonstrated that CD4+CD25+ Tregs accumulating at the site of infection were responsible for promoting pathogen persistence that was mediated by IL-10 dependent and IL-10 independent mechanisms (Belkaid *et al.*, 2002). In addition, depletion of CD4+CD25+ Tregs via anti-CD25 monoclonal antibody (mAb) therapy resulted in enhanced clearance of *L. major* parasites (Mendez *et al.*, 2004). Similarly, following infection of B6 mice with a Th1 resistant strain of *L. major* (i.e. not susceptible to a dominant protective Th1 response), resistance and parasite survival in the host is associated with the induction of CD4+CD25+FoxP3+ Tregs (Anderson *et al.*, 2005). Depletion of Tregs with an monoclonal antibody (mAb) targeted against CD25 enhanced parasite clearance. In the same study, Anderson *et al.*, (2005), also identified CD4+CD25- T cells as a major source for IL-10 during infection. In summary, the source of IL-10 that limits the immune response to pathogens may come from other non-Treg sources including Th1 cells or Th2 cells, B cells, macrophages and DC.

1.5.3.5. Tregs and MTb:

1.5.3.5.1. Tregs in human MTb studies:

To date, data on the role of Tregs during MTb infection in both the human and mouse model has been limited. Recent studies of PBMC from human TB patients have identified an increase in FoxP3 mRNA expression (Guyot-Revol *et al.*, 2006)

and by flow cytometry, (Ribeiro-Rodrigues *et al.*, 2006) have reported an increase in the percentage of CD4+CD25+FoxP3+ Tregs that have suppressive activity *in vitro*. The findings suggest that Tregs are present in TB infected individuals and are functioning to limit the immune response.

1.5.3.5.2. Tregs in murine MTb studies:

Quinn *et al.*, (2006) have shown by flow cytometry that following intranasal infection with mycobacterial BCG there is an increase in the total number of CD4+CD25+FoxP3+ Tregs. In addition, using an over simplified model for depletion of Tregs by treating mice with the anti-CD25 mAb, showed no effect on bacterial burdens or histology. However CD4+ T cell derived IFN-gamma was enhanced in mice that received anti-CD25 mAb. In the same study, Quinn *et al.*, (2006) also presented a limited amount of data demonstrating that depletion of Tregs had no effect on clearance of MTb. The role of Tregs during MTb (which we address during murine MTb infection in chapter 4), is still in its infancy and warrants further investigation.

Chapter 2: Materials and Methods.

2.1. Mice:

Specific pathogen free BALB/c, BALB/c IL-10 deficient (BALB/c.*Il10*$^{-/-}$), C57Bl/6 (B6), and B6 recombination-activating gene (*Rag*) 1 deficient (B6.*Rag1*$^{-/-}$), were bred and housed under specific pathogen free conditions at the National Institute for Medical Research (NIMR). B6.*Il10*$^{-/-}$, B cell deficient (B6.$\mu M T^{-/-}$) and BALB/c.*Rag2*$^{-/-}$ mice were a kind gift from J. Langhorne (NIMR). For *in vivo* experiments with MTb, mice were housed under bio-safety containment level 3 conditions. All mice were between 8-10 weeks of age at the start of experiments.

2.2. Reagents:

Culture medium used for all *in vitro* applications was RPMI 1640 (BioWhittaker) with the following supplements: 5% heat inactivated fetal calf serum (FCS) (Labtech International), 2-mercaptoethanol (0.05 mM; Sigma), penicillin (100U/ml; BioWhittaker), streptomycin (100µg/ml; BioWhittaker), L-glutamine (2mM; Sigma), sodium pyruvate (1mM; BioWhittaker), HEPES (10mM; BioWhittaker), GM-CSF (10ng/ml; Schering-Plough), Flt3-ligand (100ng/ml; R & D systems). Culture medium plus the above additives is referred to as cRPMI.

2.2.1. TLR ligands:

To stimulate DC *in vitro* CpG-B DNA 1668 ISS (5'-TCCATGACGTTCCTGATG CT; Invitrogen Life Technologies) was used. For *in vivo* challenge of mice CpG-B DNA 1018 ISS (5'-TGACTGTGAACGTTCGAGA; Invitrogen Life Technologies) was used.

2.2.2. Mycobacteria and Mycobacterial antigens:

All experiments using MTb were performed under bio-safety containment level 3 conditions. MTb H37Rv was used for *in vitro* infections (H37Rv sub-strain as indicated), for *ex vivo* re-stimulations BCG (Danish strain [$3.3x10^5$ CFU/ml]; Statens Serum Institute (SSI) - batch #101063), Tuberculin PPD (8-10μg/ml; SSI - Batch RT49) and plate bound anti-CD3 mAb (5μg/0.5ml coated overnight; 145.2C11 BD) were used to stimulate cells.

2.2.3. In vivo MTb studies:

MTb H37Rv was used for *in vivo* infections. H37Rv London School of Hygiene and Tropical Medicine (LSHTM) strain was cultured by seeding 100ul of MTb stock in 10ml aliquots of 7H9 culture broth (in 50ml Falcon polypropylene tubes; BD) supplemented with Tween-80 (0.05%) and 10% oleic acid-albumin-dextrose catalase (OADC; BD Difco #212240) and incubated as a static culture at 37°C. The growth of MTb was monitored by optical density at 600nm (OD_{600}) and harvested when an OD of approximately 0.6 was reached. Final MTb stocks were washed twice with saline (25 minutes at 3000 rpm) and aliquoted as 250μl aliquots into pre-labelled 1 ml freezing vials then stored at –80°C. To ascertain the number of viable colony forming units (CFU) in the H37Rv stock, aliquots were thawed, serially diluted then plated on 7H11 agar plates supplemented with OADC (10%) and read after 16-18 days. To determine whether the MTb stock was free from other contaminating organisms stocks were checked by plating 250μl on blood agar plates (5% sheep's blood; BD) for 5 days.

Intraperitoneal (I.P.) injections of immune-modulatory antibodies were performed weekly (or as indicated) in sterile PBS Dulbeccos (Gibco) as listed below (Table 2.1).

Table 2.1: Immune-modulatory antibodies:

Antigen recognised:	*Clone:*	*Species origin:*	*IgG class*	*Dose /mouse:*	*Source:*
beta-galactosidase	GL113	Rat	IgG1	As indicated	DNAX
IL-10 receptor (IL-10R)	1B1.3A	Rat	IgG1	0.5mg	DNAX
CD25 (IL-2R-alpha)	PC61	Rat	IgG1	0.5mg	NIMR
Viral hexon	TC31.27F11	Mouse	IgG1	1.5mg	Harlan
TGF-beta1/b2/b3	1D11	Mouse	IgG1	1.5mg	R&D
Plasmacytoid pDC	120G8	Rat	IgG1	1mg	G. Trinchieri

2.2.4. Cytometry reagents:

For analysis of cell surface markers from both *in vitro* and *in vivo* experiments, cells were pre-treated for 10 minutes with anti-FcγII/FcγII (anti-CD16/CD32) to minimise aspecific antibody binding. Staining was performed in FACS buffer (Dulbeccos PBS supplemented with 1%FCS, penicillin [100U/ml] and streptomycin [100μg/ml]) on ice with antibodies as described below (Table 2.2). Cells were then washed with FACS buffer (1200rpm, 7 minutes) and acquired on a FACS Caliber flow cytometer (BD Biosciences). The data was analysed using FlowJo version 8 (Treestar) software.

Table 2.2: Cytometry reagents: concentrations, clones and origins:

Antigen recognised	*Clone*	*Fluoro-chrome*	*Species origin and IgG class*	*Working concentration*	*Source*
FcγIII/FcγII (CD16/CD32)	2.4G2	----------	Rat IgG2a κ	1μg/ml	NIMR
CD4	RM4-5	FITC	Rat IgG2a κ	2μg/ml	Ebioscience
B220	RA3-6B2	FITC	Rat IgG2a κ	5μg/ml	Ebioscience
CD8	53-6.7	PE	Rat IgG2a κ	2μg/ml	Ebioscience
CD25 (IL-2R-alpha)	PC61	PE	Rat IgG1 λ	2μg/ml	Ebioscience
CD11c	HL3	PE	Hamster IgG1 λ	2μg/ml	BD
F4/80	BM8	PE	Rat IgG2a κ	2μg/ml	Ebioscience
CD11b	M1/70	APC	Rat IgG2b κ	1μg/ml	Ebioscience
GR-1 (Ly6C)	RB6-8C5	APC	Rat IgG2b κ	1μg/ml	Ebioscience
FoxP3	FJK-16s	APC	Rat IgG2a κ	1μg/ml	Ebioscience
CD25 (IL-2R-alpha)	7D4	Biotin	Rat IgM κ	1μg/ml	BD
Plasmacytoid pDC	120G8	ALEXA-488	Rat IgG1 κ	0.5μg/ml	NIMR
Isotype	IgG1	ALEXA-488	Rat IgG1 κ	As indicated	NIMR
Isotype	IgG1	PE	Rat IgG1 κ	As indicated	BD
Isotype	IgG2a	APC	Rat IgG2a κ	As indicated	Ebioscience

2.2.5. Immunoassay:

Protein analysis of supernatants from both *in vitro* and *in vivo* experiments were carried out by enzyme linked immuno-adsorbent assay (ELISA) using 96 well flat bottom plates (Nunc); as detailed below (Table 2.3). For quantification of cytokine production, cell free supernatants were collected at the time-points indicated after infection/stimulation and analysed by ELISA. A commercially available ELISA kit (Ebioscience) was used to determine IL-12p70 and TNF concentrations in sample supernatants, both cytokine ELISA's were performed according to manufacturers instructions. Antibody capture and detection pairs were used for the cytokines IL-10, IL-12p40 and IFN-alpha. Supernatants that were potentially MTb contaminated were gamma-irradiated (20 kGrays) then plated on to 7H11 agar plates supplemented with 10% OADC for 4 weeks to check sterility. In addition, *Listeria monocytogenes* was used as a fast growing control to confirm sufficient radiation exposure.

Table 2.3: ELISA reagents: concentrations, clones and origins:

ELISA:	*Standards: Starting concentration in ( )*	*Capture antibody:*	*Detection antibody:*	*Detection limit (substrate):*
IL-10	rmIL-10 (10ng/ml; DNAX)	2A5 (5μg/ml; DNAX)	SXC-1 (250ng/ml; Pharmingen)	40 pg/ml (TMB)
IL-12p40	rmIL-12 (50ng/ml; R & D systems)	C15.6 (5μg/ml)	C17.8 (1μg/ml)	50 pg/ml (ABTS)
IL-12p70	IL-12p70 kit (10ng/ml; Ebioscience)	Monoclonal anti p35	Monoclonal anti p40	20 pg/ml (TMB)
TNF	TNF kit (10ng/ml; Ebioscience)	IF3F3D4	MP6-XT3 MP6-XT22	20 pg/ml (TMB)
IFN-alpha	rmIFN alpha (10,000 U/ml; DNAX)	F18 (5μg/ml; HyCult Biotechnology)	Polyclonal rabbit anti-mouse IFN alpha. (5ug/ml; PBL Biomedical)	40 pg/ml (ABTS)

2.3. Derivation of mouse BM APC:

2.3.1. Derivation of GM-CSF cultured BM DC:

CD11c+ DC were generated using cRPMI supplemented with recombinant GM-CSF as described previously (Inaba *et al.*, 1992). Cells were isolated from the BM by flushing femurs and tibias with ice-cold cRPMI. Centrifuge the cells once (1400 rpm, 5 minutes) and then re-suspend in 0.83% ammonium chloride to lyse red blood cells (0.5ml/1x10^7 cells). Wash the cells twice with cRPMI, re-suspend in 10 ml cRPMI and count. BM cells were then plated out in six well tissue culture plates (Costar) at 10^6 cells/ml (total 5 ml/well) in cRPMI supplemented with 10 ng/ml GM-CSF. After 2 days, remove the culture medium and wash each well with 5ml cRPMI to remove non-adherent cells. 5ml cRPMI (supplemented with 10 ng/ml GM-CSF) was then added to each well. After 4 days the non-adherent cells were again discarded and 5ml cRPMI (supplemented with 10ng/ml GM-CSF) was then added to

each well. On day 6, non-adherent cells were recovered. Cells were then cultured ($0.5x10^6$ DC/ml) for a further 24hrs with cRPMI (supplemented with 10 ng/ml GM-CSF), subsequently harvested, counted and used in stimulations. The purity was always >70% CD11c+ by flow cytometry.

2.3.2. Derivation of Flt-3L cultured BM derived DC:

Plasmacytoid pDC were generated by culturing BM cells in cRPMI containing 100ng/ml Flt3-ligand at $1x10^6$ cells/ml in 12 well flat-bottom plates (2ml/well) for 10 days. On day 5, 1ml of medium was removed and 1ml of fresh cRPMI containing Flt3-ligand was added at a final concentration of 100ng/ml. Plasmacytoid pDC were sorted on either a) CD11c+ CD11b- B220+ or b) CD11c+ 120G8+. The purity was always >99% by flow cytometry.

2.3.3. Derivation of macrophages from BM:

F4/80+ macrophages were generated using cRPMI supplemented with M-CSF derived from L-929 cell conditioned medium. BM cells were isolated as above; the BM cells were cultured with cRPMI with M-CSF (20% L-929 conditioned medium) and plated out on bacteriological Petri-dishes at $0.5x10^6$ cells/ml (total volume was 8ml per dish). After 4 days, 10ml of cRPMI containing M-CSF (20% L-929 conditioned medium) was added to each dish. On day 7, non-adherent cells were discarded; 10mls of ice cold PBS was added to each dish and then incubated at +4C for 15 minutes. Macrophages were harvested, counted and used for further experiments. The purity was always >90% F4/80+ by flow cytometry.

2.3.4. Determination of APC purity after culture in vitro:

The purity of GM myeloid DC generated as described above (2.3.1) was determined by cytometric analysis of CD11c expression. Similarly, BM macrophages were stained for expression of the macrophage-restricted marker F4/80. $2x10^5$ enriched cells were plated out in 96 well round bottom plates. The cells were kept on ice where possible. BM cultured cells were pre-treated with anti-FcγII/FcγII (anti-CD16/CD32) to minimise aspecific antibody binding. BM myeloid DC and BM macrophages were stained on ice for 30 minutes with CD11c-PE (2μg/ml) or F4/80-PE (2μg/ml) respectively. The cells were then washed with FACS buffer (2000rpm, 20 seconds) and acquired on a FACS Caliber flow cytometer (BD Biosciences). The data was analysed using FlowJo version 8 (Treestar) software.

2.4. Derivation of plasmacytoid pDC from the spleen:

Splenic plasmacytoid pDC for *in vitro* stimulations were isolated by MACS separation from splenocyte suspensions using the Miltenyi CD11c+ beads. Spleens were aseptically removed and collected in cRPMI. Spleens were treated with liberase Cl (30 mins, 37°C, 2% CO_2). The organs were then homogenised by passing through a 70μm sieve with the piston from a 2ml syringe. Centrifuge the cells once (1400 rpm, 5 minutes) and then re-suspend in 0.83% ammonium chloride to lyse red blood cells (0.5ml/$1x10^7$ cells). Wash the cells twice (1400 rpm, 5 minutes) and put into FACS buffer. Using the Miltenyi CD11c+ beads following the manufacturers instructions CD11c+ cells should be positively selected. Wash the cells and count and re-suspend in FACS buffer at $20x10^6$ cells/ml in the following antibodies B220-FITC (5μg/ml), CD11c-PE (2μg/ml), GR1-APC (1μg/ml) for 20 mins on ice. Wash

the cells twice in FACS buffer (1300rpm, 7 mins). Re-suspend at $20x10^6$ c/ml in sorting buffer containing 1 μg/ml propidium iodide and to remove cell clumps pass through a 40 μm sieve. Splenic plasmacytoid pDC were sorted on CD11c+ CD11b- B220+. The purity was always >98% by flow cytometry.

2.5. *In vitro* infection of APC:

2.5.1. Infection of myeloid APC:

BM DC and macrophages were plated in antibiotic free cRPMI at $0.5x10^6$ cells/well in 48 well. MTb was added directly to the wells at a multiplicity of infection (MOI) of either 1:1 or 5:1 (MTb:APC) and the cells incubated at 37°C (5% CO_2) until harvest. The number of bacilli in the inoculum was determined by serial dilutions in PBS before plating on 7H11 agar plates supplemented with OADC (10%). The MTb was left in the wells for the duration of the experiment. The cells were harvested at the appropriate time-points post infection and supernatant collected. For determination of CFU in macrophages, the wells were washed twice with PBS to remove residual extra-cellular bacteria and lysed as described below (2.5.2). For BM DC, cells were collected, spun (1200 rpm, 7 mins) and the supernatant collected. The pellet was washed twice (1200rpm, 7 mins) with cRPMI (without antibiotics) to remove residual extra-cellular bacteria. DC cell pellets were lysed and used for CFU determination as described below (2.5.2).

2.5.2. Assessment of MTb in infected APC:

Infected cell pellets were treated with 1ml saponin (0.2%; Sigma) and left for one hour at room temperature. Serial dilutions were then performed in PBS and plated out on 7H11 agar plates supplemented with OADC (10%) to determine CFU. After 16-18 days at 37°C, visible colonies could be counted and bacterial load per well calculated.

2.6. *In vivo* MTb infections:

2.6.1. MTb infection of mice:

MTb H37Rv was thawed from pre-aliquoted stocks and used to infect mice either intravenously (I.V.) or by aerosol exposure; the infection inoculum was serially diluted on the day of infection to determine the exact MTb dose. For I.V. infections, mice were injected with the desired number of H37Rv CFU in a total volume of 200µl via the lateral tail vein (as specified in Figure legends). For aerosol infections, approximately $1x10^7$ CFU was aerosolised over a period of 30 minutes in a three-jet Collison nebuliser unit. Each mouse received approximately 50-100 CFU to the lungs as confirmed by bacterial burdens assessed on day 1 post infection. For infections using immuno-deficient mice, BALB/c.*Rag2*$^{-/-}$ were infected I.V. with $1x10^6$ CFU and monitored daily for signs of disease/pathology, mice that exhibited signs of moderate disease were killed and the "survival" time noted.

2.6.2. Determination of bacterial burdens within infected organs:

For both I.V. and aerosol infections, mice were taken at the time-points indicated and organs prepared as described below. Mice were killed by exposure to carbon dioxide and death was confirmed by cervical dislocation. The spleen and lungs were aseptically removed and put into 15ml tubes (Corning) containing 5ml of PBS. The organs were homogenised by passing through a 70μm sieve with the piston from a 2ml syringe. To determine the bacterial levels within infected organs, serial dilutions were made in PBS then 100μl of each dilution was spread onto 7H11 agar plates supplemented with OADC (10%). After 16-18 days at 37°C, visible colonies were counted and the bacterial load per organ calculated.

2.6.3. Neutralisation of cytokines and/or depletion of target cells by antibody therapy in vivo:

A summary of immune modulatory antibodies with concentrations, clones and origins is presented in Table 2.1 above.

2.6.3.1. Cytokine neutralisation:

To block the IL-10R, anti-IL-10R mAb (1B1.3A, 0.5mg/mouse in PBS) was injected via the I.P. route at the time points indicated. To block TGF-beta, anti-TGF-beta 1, 2, 3 mAb (1D11, 1mg/mouse in PBS) was injected via the I.P. route once weekly from the day of infection or as indicated in Figure legends.

2.6.3.2. Target cell depletion:

To deplete CD25+ Tregs, mice were treated with anti-CD25 (PC61, 0.5mg/mouse in PBS) at day -4, -2, 0 and 7 post infection. Flow cytometry analysis of Tregs (CD4+CD25+FoxP3+) at day 0 showed a >80% depletion. For depletion of plasmacytoid pDC, mice were treated with 120G8 mAb (1mg/mouse in PBS) at day -1, 0, 7, and 14 post infection. Flow cytometry analysis of plasmacytoid pDC (CD11c+GR1+B220+) at day 0 showed a >80% depletion.

Control mice for all depletion experiments using anti-IL-10R (1B1.3A), anti-CD25 (PC61) and 120G8 were given the same antibody regimen and received an appropriate dose of irrelevant rat IgG1 mAb (GL113; anti-beta-galactosidase). For neutralisation of TGF-beta, control mice given the same antibody regimen of irrelevant mouse IgG1 antibody (TC31.27F11; anti-viral hexon).

2.6.4. Functional analysis of immune cells obtained from MTb infected mice:

All *ex vivo* stimulations were performed in triplicates in Costar 24 well flat bottom plates in a final volume of 1.5 ml. Spleen and lung homogenates were treated with ammonium chloride (0.83%) to lyse red blood cells. Cells from the spleen ($5x10^6$/1.5ml), and lung ($1x10^6$/1.5ml) were stimulated with either BCG ($5x10^5$ CFU/1.5 ml), Tuberculin PPD (8-10µg/ml) or plate bound anti-CD3 mAb (5µg/0.5ml; coated overnight). Cell cultures were incubated at 37°C (5% CO_2). Supernatants were harvested as indicated in figure legends then irradiated as described above.

2.6.5. FACS analysis of cells from MTb infected mice:

Where possible all staining was carried out on ice. Whole spleen ($5x10^6$ cells) and lung ($1x10^6$ cells) suspensions were stained in 5ml FACS tubes for 10 minutes with 1ug/ml anti-FcγIII/FcγII (anti-CD16/CD32). Cells were then stained for 25 minutes with the appropriate concentration of cell surface antibodies in a total volume of 200ml. Cells were washed twice in FACS buffer then fixed in 500ml 2% formaldehyde overnight and fumigated within the class I microbiological safety cabinet at room temperature.

For determination of FoxP3 expression, cells were stained according to the manufacturers protocol. Briefly, cells were stained for CD4-FITC and CD25-PE then fix/permeabilised for 2 hours at 4°C with an Ebioscience fix/permeabilisation buffer. Cells were then further washed with permeabilisation buffer then stained with FoxP3-APC for 30 minutes. Cells were then washed twice with Ebioscience permeabilisation buffer and once with FACS buffer then fixed and fumigated as described above. All samples were acquired the next day on a FACS Calibur within the bio-safety containment level 3 suite at the LSHTM.

2.7. T cell reconstitution:

2.7.1. Purification of T cell subsets:

CD4+CD25- and CD4+CD25+ T cells for adoptive transfer were isolated by MACS separation from splenocyte suspensions using the Miltenyi CD4+CD25+ regulatory T cell isolation kit. Briefly, the spleens were aseptically removed and collected in cRPMI. The organs were homogenised by passing through a 70μm sieve with the

piston from a 2ml syringe then treated with ammonium chloride (0.83%) to remove red blood cells and re-suspended in FACS buffer. Using the Miltenyi kit; CD4+ cells were negatively enriched using lineage specific biotin-conjugated antibodies against CD8 (Ly-2), CD11b (Mac-1), CD45R (B220), CD49b (DX5), Ter-119, and anti-biotin MicroBeads were used. CD25-PE and anti-PE MicroBeads were used for the subsequent positive selection of CD4+CD25+ regulatory T cells from the enriched whole CD4+ fraction. The purity of the enriched CD4+CD25- (>90% pure) and CD4+CD25+ (>95% pure) populations was confirmed by flow cytometry.

2.7.2. T cell reconstitution of immuno-deficient Rag$^{-/-}$ mice:

Rag$^{-/-}$ mice were injected I.V. with MACS sorted CD4+CD25- T cells (>92% pure) alone or together in the presence CD4+CD25+ T cells (>95% pure) as specified. All injections of T cells were performed in PBS the day before MTb H37Rv infection.

2.8. Statistical analysis:

All data was analysed as indicated in figure legends using either a) Students *t* test or b) One-Way ANOVA combined with a Bonferroni multiple comparison post-test with differences being considered significant when $P < 0.05$.

Chapter 3: The Role of IL-10 in Regulating Protective Immunity to MTb Infection.

3.1. Determining a role for IL-10 in regulating immunity to MTb, aims of the investigation:

To address a role for IL-10 during MTb infection we sought to identify:

1) During late stages of murine MTb infection, does IL-10 play a regulatory role on bacterial clearance?

2) Is the early immune response to MTb enhanced in the absence of IL-10?

3) Does endogenous IL-10 regulate macrophage anti-mycobacterial effector function during *in vitro* MTb infection?

4) To determine a cellular source for IL-10 during early MTb infection.

3.2. Background:

Control and potential clearance of intracellular pathogens such as MTb requires a strong pro-inflammatory response dependent on TNF and the induction of IFN-gamma through the Th1 polarising cytokine IL-12 (Flynn *et al.*, 2001, Baumann *et al.*, 2006). However, this may lead to host pathology due to over-exuberant effector responses. The presence of regulatory factors may benefit the host by limiting collateral damage but may inadvertently promote pathogen persistence. It has been demonstrated in infectious diseases that the presence of IL-10 prevents pathogen clearance and promotes parasite persistence but can also protect against immuno-pathology (Moore *et al.*, 2001).

Studies in the mouse by North *et al.*, (1998) and Jung *et al.*, (2003) have shown that IL-10 has no apparent role to control the immune response or affect bacterial clearance during early MTb infection. However, these observations are in contrast

to Roach *et al.*, (2001b), who showed a transient decline in bacterial load early during infection in the absence of IL-10. Turner *et al.*, (2002) using a transgenic mouse model where IL-10 is under control of the IL-2 promoter, have shown that over-expression of IL-10 enhances bacterial burdens during the latter stages of MTb infection. However, this approach may be seen as non-physiological as IL-10 is not under the control of its natural promoter.

In human studies, Gong *et al.*, (1996) have shown that neutralisation of endogenous IL-10 in PBMC cultures from active TB patients increased IFN-gamma responses to the heat-killed MTb. Similarly, Boussiotis *et al.*, (2000) have also reported a similar observation on T cell proliferation when PBMC from anergic TB patients were stimulated with PPD in the absence of IL-10. Gerosa *et al.*, (1999) have shown that in the BAL of active TB patients CD4+ T cells are present producing both IFN-gamma and IL-10. Taken together these studies in humans suggested that IL-10 may suppress the clearance of MTb and contribute to chronic infection. However, this cannot not be demonstrated in human studies at this stage and has only been inferred.

Thus the role of IL-10 in suppressing the immune response to MTb and affecting bacterial clearance can only be demonstrated in experimental models. To resolve the contradictory data in the mouse we have examined the role of IL-10 in immune responses to MTb infection. More specifically, we determined whether at an early (acute) or late (chronic) stage of MTb infection, endogenous IL-10 suppressed a protective Th1 response (characterised by IFN-gamma), and consequently the clearance of MTb.

3.3. The immune response to different strains of MTb H37Rv: diverging roles for IL-10.

The conflicting murine data with regard to the role of IL-10 in regulating the early response to MTb and studies by Turner *et al.*, (2002) using an IL-10 transgenic mouse suggested a role for IL-10 late during chronic MTb infection. We first sought to identify a role for IL-10 at a late stage of infection with the MTb strain H37Rv NIMR. We also performed this study on an independent strain of H37Rv from the London School of Hygiene and Tropical Medicine (LSHTM) to ensure standardisation between strains of H37Rv. To selectively block IL-10 signalling *in vivo*, a monoclonal antibody (mAb) to the IL-10 receptor (anti-IL-10R mAb) was administered after MTb infection was established. Studies in the literature on the role of IL-10 in regulating the early immune response to MTb to have thus far used *Il10*$^{-/-}$ mice which failed to establish a role for IL-10 during the late stages of infection.

3.3.1. IL-10 functions during late MTb infection with H37Rv NIMR to regulate bacterial clearance:

To address a role for IL-10 in regulating the immune response to MTb at different stages after infection; *i)* BALB/c mice were infected I.V. with H37Rv NIMR for 123 days to establish a chronic infection (Fig. 3.1A) as demonstrated by a plateau in bacterial burdens within infected organs. When a chronic infection was established mice were treated once weekly from day 90 post infection with either anti-IL-10R mAb (0.5mg/mouse) or PBS (control) and at day 123 the experiment was terminated.

Bacterial burdens (CFU) in the lungs and spleen were monitored during the course of infection and determined as discussed in Materials and Methods. Treatment of infected mice with either PBS or an isotype control IgG (GL113), have shown similar effects on bacterial burdens *in vivo* (Boonstra A., O' Garra A., unpublished observations).

Infection with H37Rv NIMR in immuno-competent mice exhibited an unconventional pattern of bacterial growth compared to the literature, where the total bacterial counts in the spleen and lung did not converge and stabilise during the latter stages of infection (Fig. 3.1A). When intervening with anti-IL-10R mAb at day 90 post MTb infection, the bacterial loads were reduced by 2-Log_{10} in the spleen ($P < 0.0001$) and 1.5-Log_{10} in the lung ($P < 0.0001$) compared to control treated MTb infected mice (Fig. 3.1B and C respectively). To examine the effect on cytokine production following IL-10 neutralisation *in vivo,* cell suspensions from infected mAb treated mice were re-stimulated *ex vivo* in the presence of the mycobacterial antigens BCG ($3.3x10^5$ CFU/ml) or PPD (10μg/ml). The decrease in bacterial load following anti-IL-10R mAb treatment *in vivo* was associated with an increase in Th1 cytokines as stimulation of splenocytes from anti-IL-10R mAb treated mice gave a significant increase in IFN-gamma ($P < 0.05$) (Fig. 3.2A and B) and TNF (Fig. 3.2C and D) compared to MTb infected control treated mice. Stimulation of whole cell suspensions obtained from infected mice in the absence of mycobacterial stimuli did not produce any detectable levels of cytokines IFN-gamma or IL-12 (as demonstrated in Fig. 3.8).

In a second experiment when intervening in BALB/c mice once weekly with anti-IL-10R mAb at day 165 post MTb infection (Fig. 3.3A), the bacterial burdens were approximately five fold higher in the spleen and the lungs as compared to the previous intervention at day 90 in un-manipulated control infected mice (Fig. 3.1A). The effect of anti-IL-10R mAb treatment at this time was less pronounced giving only a 0.5-Log_{10} reduction in the spleen ($P < 0.0001$) (Fig. 3.3B) with no apparent reduction in the lung (Fig. 3.3C) as compared to control treated mice. Similarly, the pattern of bacterial growth within infected organs (Fig. 3.3A) was identical to the previous study (Fig. 3.1A), where the total bacteria burdens in the spleen and lung did not converge during the latter stages of infection, as compared to bacterial growth curves for H37Rv infection reported broadly in the literature (North, 2004).

To determine the cytokine responses in mice treated with anti-IL-10R mAb, whole splenocyte cell suspensions were re-stimulated *ex vivo* with BCG or PPD. At this stage during infection there was no enhancement of IFN-gamma (Fig. 3.4A and B) or TNF (Fig. 3.4C and D) in splenocytes derived from mAb treated mice in response to either BCG or PPD. However, the levels of inducible IFN-gamma at this time were 20-fold higher at this late stage during infection as compared to the previous experiment at day 123 (Fig 3.2A and B). In addition, the levels of IL-10 present in culture supernatants following *ex vivo* PPD and BCG re-stimulation at either day 123 (data not shown) or day 205 (Fig. 3.4E and F) did not correlate with the levels of IFN-gamma.

3.3.2. Regulation of bacterial load during late infection with MTb H37Rv LSHTM strain is not dependent on IL-10:

To further evaluate a role for IL-10 in suppressing immune responses to MTb at a late stage of infection and the observation that the *in vivo* bacterial growth curves observed for H37Rv NIMR were in contrast to those reported in the literature, we verified our findings with an independent isolate of MTb named H37Rv LSHTM. Interestingly, both the LSHTM strain and NIMR strain had originated from the same source as a gift from S. Cole (Pasteur Institute, Paris).

To repeat the experiments with the H37Rv NIMR when anti-IL-10R treatment had an effect on reducing bacterial levels, BALB/c mice were infected *i)* I.V. for 97 days (Fig. 3.5A) or *ii)* via aerosol for 90 days (Fig. 3.5B), before mAb treatment was started. Mice were treated once weekly I.P. with anti-IL-10R mAb (0.5mg/mouse) or PBS (control) when at day 133 (Fig. 3.5A - I.V.) or day 125 (Fig. 3.5D - aerosol), mice were killed and bacterial loads in the spleen (Fig. 3.5B and E) and lungs (Fig. 3.5C and F) were determined.

In contrast to the growth curves observed with H37Rv NIMR, the growth curves for H37Rv LSHTM in infected organs were similar to the infection curves published in the literature where both the spleen and lung converged and stabilised relatively early (day 30-40) after infection. Neutralisation of IL-10 signalling late after I.V or aerosol infection with H37Rv LSHTM strain did not influence the infection outcome. This finding was in contrast to anti-IL-10R mAb treatment starting day 90

during late I.V. infection with the H37Rv NIMR strain (Fig. 3.1B and C). The *in vivo* bacterial growth curves observed for H37Rv NIMR and H37Rv LSHTM in our experiments are compared in Fig. 3.5. Our findings may reflect the differential induction of IL-10 during infection or differing virulence between the H37Rv strains.

3.3.3. Does the H37Rv strain and infection dose affect the regulation by IL-10 in vivo?

We show here that IL-10 appears to play a role during the latter stages of infection with H37Rv NIMR but not H37Rv LSHTM. The potential reasons for the observed differences in bacterial levels following anti-IL-10R mAb treatment could be attributed to *i)* H37Rv strain, or *ii)* that the initial infection dose of H37Rv NIMR was lower than that of H37Rv LSHTM. (However, this could not be further addressed with H37Rv NIMR as the collaborators original stock of H37Rv NIMR was no longer available).

Using a freshly grown batch of H37Rv NIMR and H37Rv LSHTM, studies within the laboratory have identified a vast difference in virulence between the two H37Rv strains NIMR and LSHTM following infection of immuno-compromised *Rag*$^{-/-}$ mice (Boonstra, A., Holman, M., O'Garra, A., unpublished data). BALB/c.*Rag2*$^{-/-}$ were infected I.V. with $1x10^6$ CFU and monitored daily for signs of disease/pathology, mice that exhibited signs of moderate disease were killed and the survival time noted. The differences observed between the H37Rv strains were striking; mice infected with H37Rv LSHTM had a mean survival time of 25 days compared with

46 days for H37Rv NIMR. For the reason of reduced virulence of H37Rv NIMR, we focussed our studies from thence solely on H37Rv LSHTM as the growth curves of this isolate were more reflective of what has been reported in the literature.

To determine whether the lack of effect when neutralising IL-10 late during infection with H37Rv LSHTM was because the infecting dose was too high, we administered anti-IL-10R mAb late after infection with different doses of H37Rv. BALB/c mice were infected I.V. with A) 23,350 CFU, B) 161,000 CFU, C) 234,000 CFU and D) 1,812,000 CFU H37Rv LSHTM strain. Starting at day 35, mice received weekly injections I.P. with either anti-IL-10R mAb (0.5mg/mouse) or PBS control. At day 77, mice were killed and the bacterial levels in the spleen and lung were determined. During the latter stages of MTb infection with H37Rv LSHTM, anti-IL-10R mAb treatment had no effect on bacterial burdens and was independent of the starting infection dose (Fig. 3.7A-D).

3.4. IL-10R blockade during early MTb infection:

3.4.1. Anti-IL-10R mAb treatment of mice during early I.V. MTb infection enhances pathogen clearance and is associated with increased production of IFN-gamma:

Thus far we have shown that following I.V. infection with H37Rv LSHTM, IL-10 played no role on regulating bacterial load late during MTb infection. As the literature to date on a role for IL-10 in regulating the immune response and hence bacterial clearance during early MTb infection in mice appears controversial, we

sought to address a role for IL-10 during early infection with H37Rv LSHTM via *a)* I.V. and *b)* aerosol infection.

a) Effect of anti-IL-10R mAb intervention early in I.V. MTb infection:

BALB/c mice received anti-IL-10R mAb (0.5mg/mouse) or control IgG I.P. on the day of MTb infection, then once weekly for four weeks (until day 28). Following infection (Fig. 3.8A) we observed a decline in CFU on day 32 in the spleen of anti-IL-10R mAb treated mice ($P < 0.05$; Fig. 3.8B). However, in the lung anti-IL-10R mAb treatment resulted in a transient 0.5-Log_{10} decline in CFU at day 22 ($P < 0.01$), but by day 32 post infection this effect was less pronounced (Fig. 3.8C). In order to understand the events resulting from neutralisation of IL-10 *in vivo* which led to this transient decrease in bacterial clearance, whole splenocytes from non-infected and MTb infected mAb treated mice were re-stimulated *ex vivo* with PPD (10μg/ml) for 72 hours after which supernatants were collected and assayed by ELISA for IFN-gamma. PPD stimulation of cells from mice treated with anti-IL-10R mAb *in vivo*, exhibited a three-fold increase in IFN-gamma production compared to MTb infected control IgG treated animals ($P < 0.0001$; Fig. 3.8D). As expected, un-infected mice did not respond to stimulation with PPD.

b) Effect of anti-IL-10R mAb intervention early during aerosol MTb infection:

To address a role for IL-10 during aerosol infection with H37Rv LSHTM, BALB/c were treated with either anti-IL-10R mAb or control IgG on day 0 before aerosol infection; mice then received the same schedule of once weekly mAb injections as described above for 5 weeks (until day 35).

Mice were killed at days 27, 35 and 60 post infection and bacterial burdens in the lungs and spleen were determined as described in Materials and Methods. Treatment of mice with anti-IL-10R mAb before (at day 0) and once weekly after aerosol exposure to H37Rv LSHTM appeared to have no effect on disease progression in either the lung (Fig. 3.9A) or spleen (Fig. 3.9B) at the time-points indicated. Similarly, aerosol infection of *Il10*$^{-/-}$ mice (BALB/c.*Il10*$^{-/-}$), showed no effect on bacterial burdens in the lung (Fig. 3.9C), however in the spleen, a slight decrease in bacterial burdens was evident but failed to reach statistical significance (Fig. 3.9D).

3.5. IL-10 blockade on *ex vivo* PPD stimulated cells obtained from MTb infected mice enhances IFN-gamma production:

Gong *et al.*, (1996) have reported that neutralisation of endogenous IL-10 in human PBMC cultures restored IFN-gamma and IL-12p40 responses to PPD. The authors thus suggested that IL-10 must be regulating the immune response to MTb and therefore limiting pathogen clearance. However, this can only be inferred from these human studies using *ex vivo* derived cells from TB patients and has yet to be addressed in experimental models.

To address whether similar findings could be demonstrated in the mouse model, we used the same experimental approach as reported in the human studies. Cell suspensions were prepared from MTb infected mice at various stages after infection and IL-10 activity was neutralised *in vitro* before stimulation with MTb antigens to determine whether IFN-gamma could be enhanced. Whole splenocytes and lung preparations from non-infected and MTb infected mice (infected I.V. with H37Rv

LSHTM for 32 days), were stimulated *ex vivo* with PPD (10μg/ml) in the presence or absence of anti-IL-10R mAb (10μg/ml). Following 72 hours of culture, supernatants were collected and assayed by ELISA for IFN-gamma and IL-12p40. Stimulation of splenocytes with PPD in the presence of anti-IL-10R gave a significant enhancement of the pro-inflammatory cytokines IFN-gamma ($P < 0.01$; Fig. 3.10A) and IL-12p40 ($P < 0.05$; Fig. 3.10B) compared with splenocytes stimulated with PPD alone. In contrast to the stimulations using whole splenocytes, stimulation of lung preparations for 72 hours did not give any detectable cytokine production (data not shown). Similarly, results have also been obtained within the laboratory using spleen preparations from mice infected with H37Rv LSHTM for more than 100 days. Enhancement of IFN-gamma induced by PPD from lung cells could also be observed when stimulations were performed in the presence of anti-IL-10R mAb (Boonstra, A., O'Garra, A., unpublished data).

Our findings demonstrate that endogenous IL-10 in splenocytes obtained from MTb infect mice, dampens the IFN-gamma response to PPD *in vitro*. This suggests that IL-10 may be regulating the immune response to MTb at this stage as was also shown in the human studies by Gong *et al.*, (1996). However, the enhancement observed in IFN-gamma production may not directly reflect / correlate with pathogen clearance *in vivo*. Since, in our mouse model of infection where mice were treated *in vivo* with anti-IL-10R mAb during the latter stages of infection with H37Rv LSHTM, IL-10 blockade had no effect on reducing bacterial load (Fig. 3.5).

3.6. IL-10 functions during *in vitro* MTb infection of macrophages to negatively regulate pro-inflammatory cytokine production, but ultimately does not influence MTb killing:

Since IL-10 acts on macrophages and DC to down regulate pro-inflammatory cytokine responses we postulated that the *in vivo* effect of IL-10 blockade was to enhance the early response to MTb infection by removing its suppressive action on macrophage pro-inflammatory cytokine production and bacterial killing. To test this *in vivo* scenario, we used a simplified *in vitro* model for MTb infection using BM derived macrophages to identify whether: a) endogenous IL-10 inhibits the production of pro-inflammatory cytokines and b) macrophage MTb killing.

Macrophages were derived from the BM of BALB/c and BALB/c.*Il10*$^{-/-}$ mice as described in Materials and Methods. BM macrophages were infected with H37Rv LSHTM strain at an MOI of 1:1 (MTb: Macrophage) and 5:1. The MTb was left in the culture wells for the duration of the infection and cell culture supernatants were collected after 6, 24 and 48 hours post infection.

In vitro MTb infection of BM macrophages from wild-type BALB/c mice for 6 hours (Fig. 3.11A) and 24 hours (Fig. 3.11B) produced low levels of TNF and IL-12p40 but no detectable levels of IL-12p70 or IL-10 (Fig. 3.11B). In contrast, H37Rv infection of BM macrophages from *Il10*$^{-/-}$ mice gave significantly higher levels of TNF compared to wild-type macrophages at 6 hours (Fig. 3.11A) showing that low levels of IL-10 were induced although un-detectable. By 24 hours, the levels of IL-12p70, IL-12p40 and TNF from BALB/c.*Il10*$^{-/-}$ macrophages were greatly enhanced compared to macrophages from wild-type mice (Fig. 3.11B) as

described by others (Hickman *et al.*, 2002). In addition, the increased cytokine production following *in vitro* infection of macrophages in the absence of IL-10 was independent of mouse strain, as B6 and B6.*Il10*$^{-/-}$ mice gave comparable cytokine results to our findings in BALB/c and BALB/c.*Il10*$^{-/-}$ mice respectively (data not shown).

Macrophages from *Il10*$^{-/-}$ mice (both BALB/c and B6) produced enhanced levels of pro-inflammatory cytokines in response to H37Rv LSHTM infection. We sought to determine whether the enhanced pro-inflammatory cytokine production due to the absence of IL-10 had any effect on macrophage anti-bactericidal killing. To address this, BM macrophages were prepared and infected as described above. Macrophages were infected for 24 hours (Fig. 3.12A) and 48 hours (Fig. 3.12B) before cells were harvested and bacterial burdens were analysed as described in Materials and Methods. MTb killing in macrophages infected with an MOI of 1:1 with H37Rv LSHTM, did not alter after 24 or 48 hours of infection and was independent of the presence of IL-10 as compared to wild-type controls (Fig. 3.12). However, following infection with H37Rv LSHTM at an MOI of 5:1, we observed a decline in macrophage intracellular bacterial burdens from 24 to 48 hours post infection that was not enhanced in the absence of IL-10 (Fig. 3.12). This data suggests that increased pro-inflammatory cytokine production as a result of the removal of IL-10, does not reliably correlate with increased macrophage MTb killing.

3.7. Isolating the source of IL-10 during early infection with MTb H37Rv LSHTM:

Our data demonstrates that IL-10 appears to play a regulatory role during early but not late during I.V. MTb infection with H37Rv LSHTM. It is well documented that IL-10 is an immuno-regulatory cytokine produced by a wide array of cell types including T cells and B cells (Moore *et al.*, 2001). We therefore sought to determine whether CD4+ T cells or B cells serve as a cellular source for IL-10 during early MTb infection.

3.7.1. T cell derived IL-10 as a negative regulator of the immune response to I.V. MTb infection:

We examined a role for T cell derived IL-10 in regulating the immune response to MTb infection by adopting a number of approaches. Firstly, we tried to identify CD4+ IL-10 producers using intracellular flow cytometry. To address this, whole splenocytes and lung preparations were prepared from MTb infected mice and re-stimulated *ex vivo* with PPD (10μg/ml) or plate bound anti-CD3 for 12 hours. IL-10 could not be detected in either organ preparation after stimulation with PPD or anti-CD3 (data not shown), although we knew IL-10 was present in the cell populations from our *ex vivo* IL-10 blocking studies. The inability to detect IL-10 could be due to the low frequency of antigen specific T cells or different cell populations producing IL-10.

Therefore to address the potential role of T cell derived IL-10 in regulating the immune response to MTb infection an adoptive transfer model was used. CD4+ T cells that contain both naïve CD4+CD25- T cells and CD4+CD25+ Tregs, were purified by magnetic separation from either wild-type BALB/c or BALB/c.*Il10*$^{-/-}$ mice. Donor cells were adoptively transferred into immuno-compromised BALB/c.*Rag2*$^{-/-}$ recipient mice the day before infection with H37Rv LSHTM. At day 22 post infection the mice were killed and bacterial burdens in the spleen (Fig. 3.13A) and lungs (Fig. 3.13B) were determined. The protection afforded by CD4+ T cells following transfer in to BALB/c.*Rag2*$^{-/-}$ could not be enhanced in the absence of IL-10. Our findings demonstrate that CD4+ T cells play a pivotal role in mediating protection to MTb infection as previously reported at this stage of infection (Flynn *et al.*, 2001).

3.7.2. The role of B cells during early I.V. MTb infection:

Our data suggested that CD4+ T cells mediate protection during early MTb infection but are not a source of IL-10. Therefore the potential role of B cell derived IL-10 was addressed using B cell deficient mice on the C57Bl/6 background (B6.$\mu MT^{-/-}$).

Wild-type B6 and B6.$\mu MT^{-/-}$ were infected I.V. with 54,000 CFU and bacterial burdens in the spleen (Fig. 3.14A) and lung (Fig. 3.14B) were monitored over a period of 52 days. Following I.V. infection B6.$\mu MT^{-/-}$ displayed a transient decrease (0.5-Log_{10}) in bacterial burdens in the spleen at day 23 post infection ($P < 0.05$; Fig. 3.12C), but by day 52 this effect had diminished (Fig. 3.14E). There was no effect in bacterial burdens in the lung at either time-point day 23 or day 52 (Fig. 3.14D and

F). In addition, low dose infection of B6 and B6.$\mu MT^{-/-}$ with 2,400 CFU I.V. also showed no significant difference in bacterial burdens (data not shown). Our findings suggest a minimal and transient role for B cells and possibly B cell derived IL-10 during the early stages of immune response to I.V. H37Rv infection.

3.8. Figures:

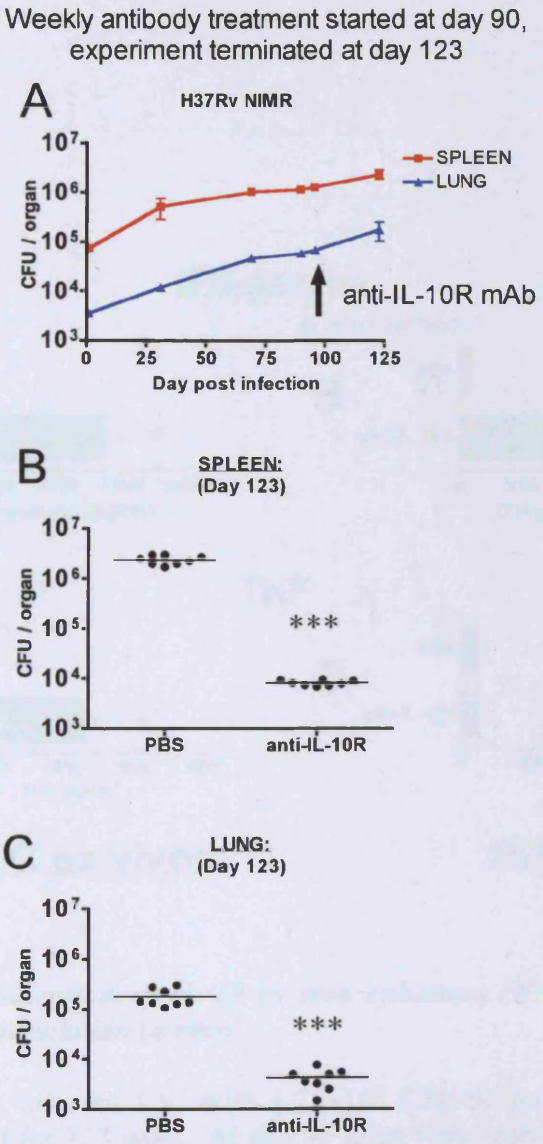


Figure 3.1: Neutralisation of the IL-10R in vivo after infection with H37Rv NIMR enhances bacterial clearance.

(A) BALB/c mice were infected I.V. with 1.75×10^6 CFU/mouse H37Rv NIMR (as determined by O.D.) for 123 days. At day 90 mice were injected once weekly I.P. with either anti-IL-10R mAb (1B1.3A; 0.5mg/mouse) or PBS (control). At day 123, mice were killed and bacterial burdens were determined in the spleen **(B)** and lung **(C)**, as described in Materials and Methods (number of mice per group = 8). Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means. The effect of IL-10R mAb treatment was tested statistically using an unpaired Students t test (***, $P < 0.0001$).

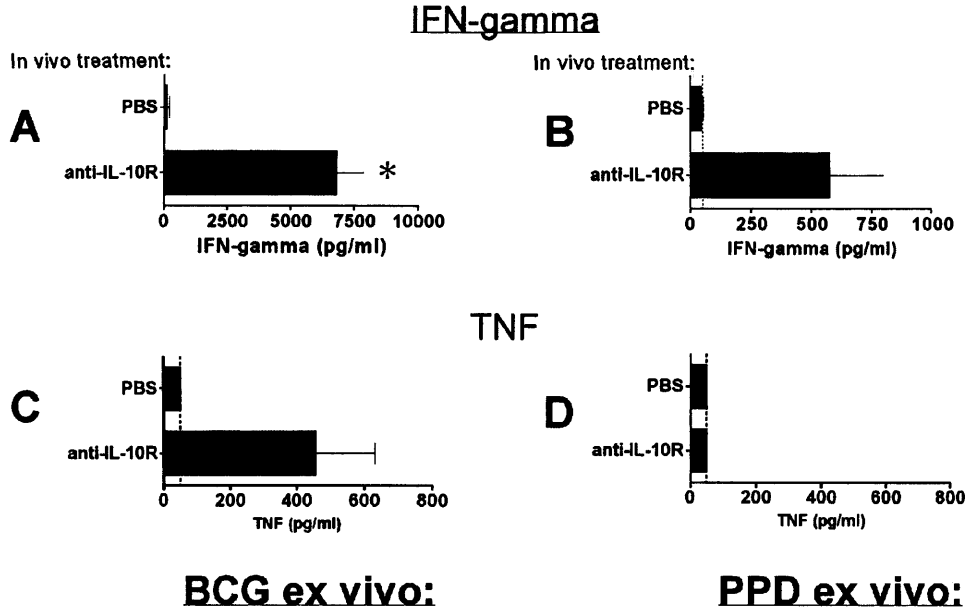


***Figure 3.2**: Neutralisation of IL-10 in vivo enhances IFN-gamma and TNF responses upon re-stimulation in vitro.*

BALB/c mice were infected I.V. with $1.75x10^6$ CFU/mouse H37Rv NIMR (as determined by O.D.) for 123 days. At day 90 mice were injected once weekly I.P. with either anti-IL-10R mAb (1B1.3A; 0.5mg/mouse) or PBS (control). At day 123, mice were killed and cell suspensions were prepared as described in Materials and Methods. Whole splenocyte cell suspensions were re-stimulated *ex vivo* with either BCG (A/C) ($3.3x10^5$ CFU/ml) or PPD (B/D) (10μg/ml). The supernatants were collected after 48 hours post stimulation and were assayed by ELISA for the production of IFN-gamma (A/B) or TNF (C/D). The effect of IL-10R mAb treatment was tested statistically using an unpaired Students t test (*, $P < 0.05$).

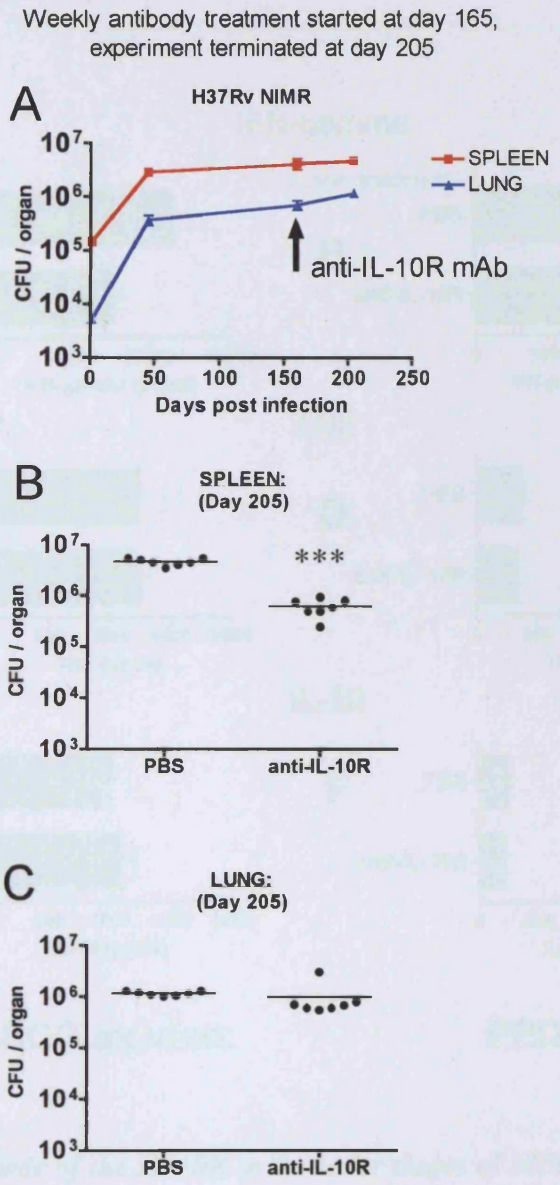


Figure 3.3: Neutralisation of IL-10 after MTb infection with H37Rv NIMR minimally enhances bacterial clearance.

(A) BALB/c mice were infected I.V. with $1.4x10^6$ CFU H37Rv NIMR (as determined by O.D.) for 205 days. Bacterial burdens were monitored in the spleen and lungs throughout the time course. At day 165 mice were injected once weekly I.P. with either anti-IL-10R mAb (1B1.3A; 0.5mg/mouse) or PBS (control). At day 205, mice were killed and bacterial loads were determined in the spleen (B) and lung (C), as described in Materials and Methods (number of mice per group = 7). Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means. The effect of IL-10R mAb treatment was tested statistically using an unpaired Students *t* test (***, $P < 0.0001$).

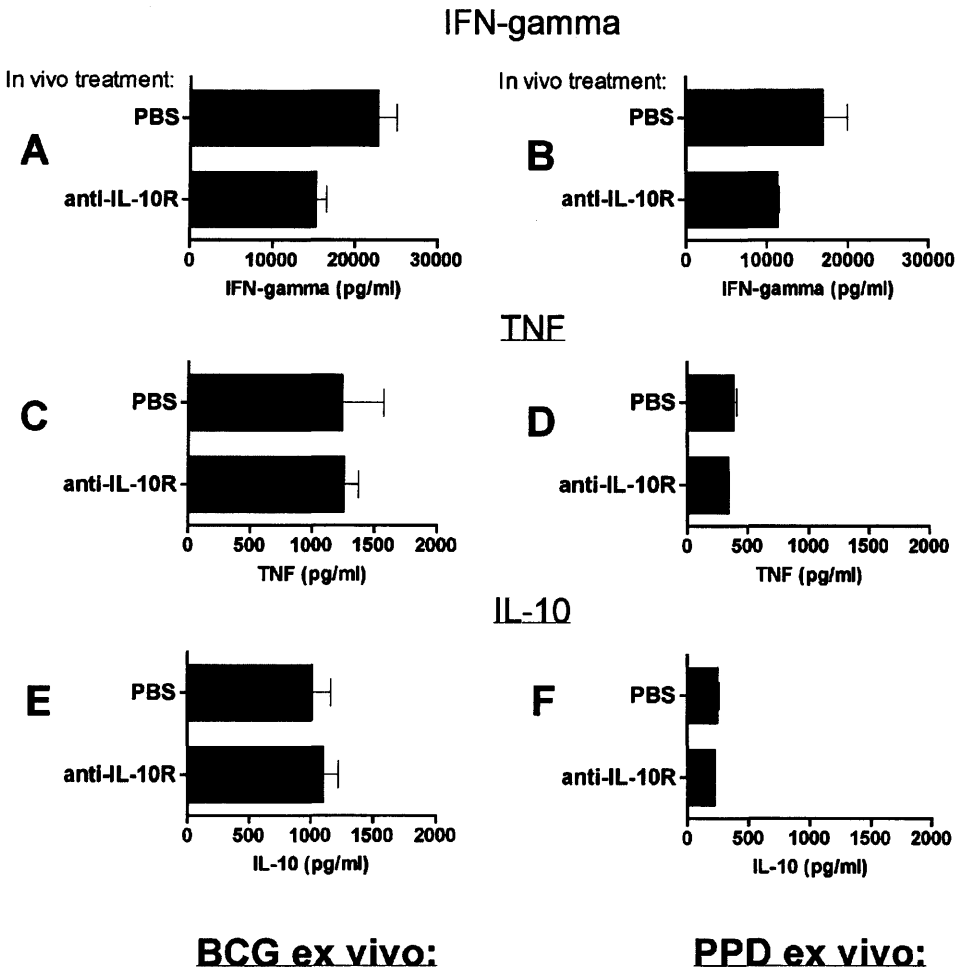


***Figure 3.4**: Blockade of the IL-10R in the latter stages of MTb infection does not enhance pro-inflammatory cytokine responses following re-stimulation ex vivo.*

BALB/c mice were infected I.V. with $1.75x10^6$ CFU/mouse H37Rv NIMR (as determined by O.D.) for 123 days. At day 165 mice were injected once weekly I.P. with either anti-IL-10R mAb (1B1.3A; 0.5mg/mouse) or PBS (control). At day 205, mice were killed and cell suspensions were prepared as described in Materials and Methods. Whole splenocytes were pooled from anti-IL-10R mAb or control treated mice and re-stimulated *ex vivo* with either BCG ($3.3x10^5$ CFU/ml) or PPD (8μg/ml). Supernatants were collected after 48 hours post stimulation and assayed for production of IFN-gamma (A/B), TNF (C/D) and IL-10 (E/F) by ELISA. The effect of IL-10R mAb treatment was tested statistically using an unpaired Students t test (*, $P < 0.05$).

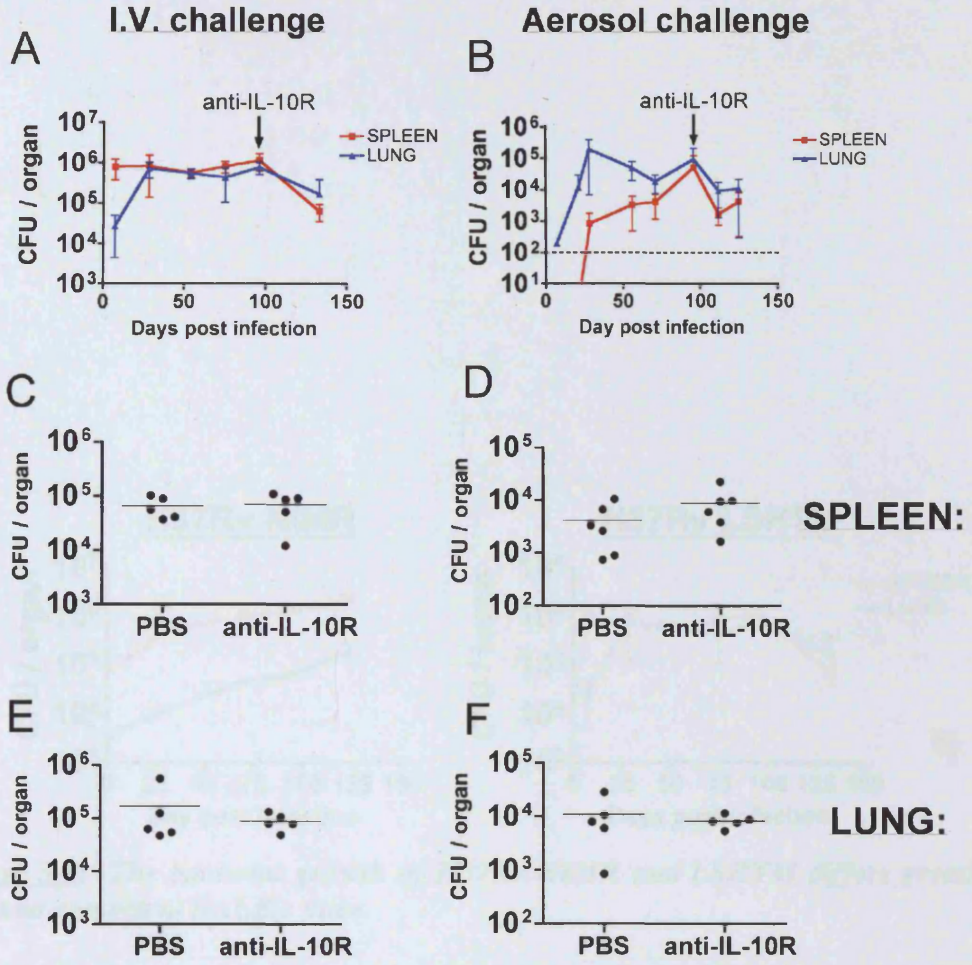


***Figure 3.5**: The absence of IL-10 signalling does not influence bacterial load following late infection with H37Rv LSHTM.*

BALB/c mice were infected with H37Rv LSHTM via **(A)** I.V. or **(B)** aerosol for a duration of 133 or 125 days, respectively. Bacterial burdens were closely monitored during infection and starting from day 97 (for I.V. infection) or day 90 (for aerosol infection) mice were injected weekly I.P. with either anti-IL-10R mAb (1B1.3A; 0.5mg/mouse) or PBS (control). At day 133 (for I.V.) or day 125 (for aerosol infection), mice were killed and bacterial burdens were determined in the spleen **(C)**/**(D)** and lung **(E)**/**(F)**, as described in Materials and Methods (number of mice per group = 5). Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means. Results shown are representative of two independent experiments.

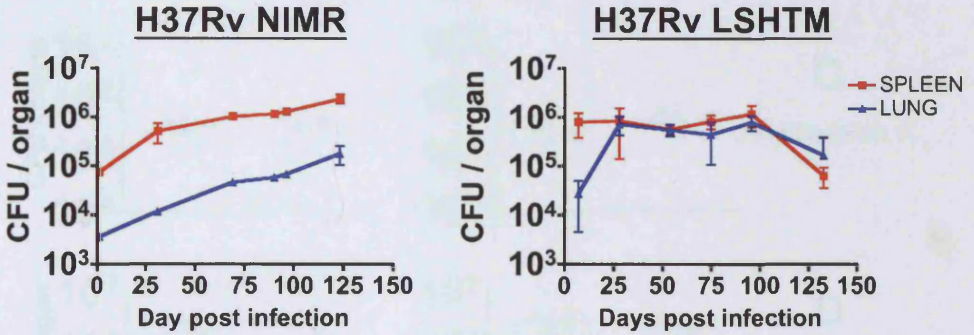


***Figure 3.6**: The bacterial growth of H37Rv NIMR and LSHTM differs greatly immuno-competent BALB/c mice.*

BALB/c mice were infected I.V. with either H37Rv NIMR ($1.75x10^6$ CFU determined by O.D.) or H37Rv LSHTM (dose unknown). During the course of infection bacterial burdens were determined in the spleen and lung at the time-points indicated as described in Materials and Methods (number of mice per group H37Rv NIMR = 8; H37Rv LSHTM = 5).

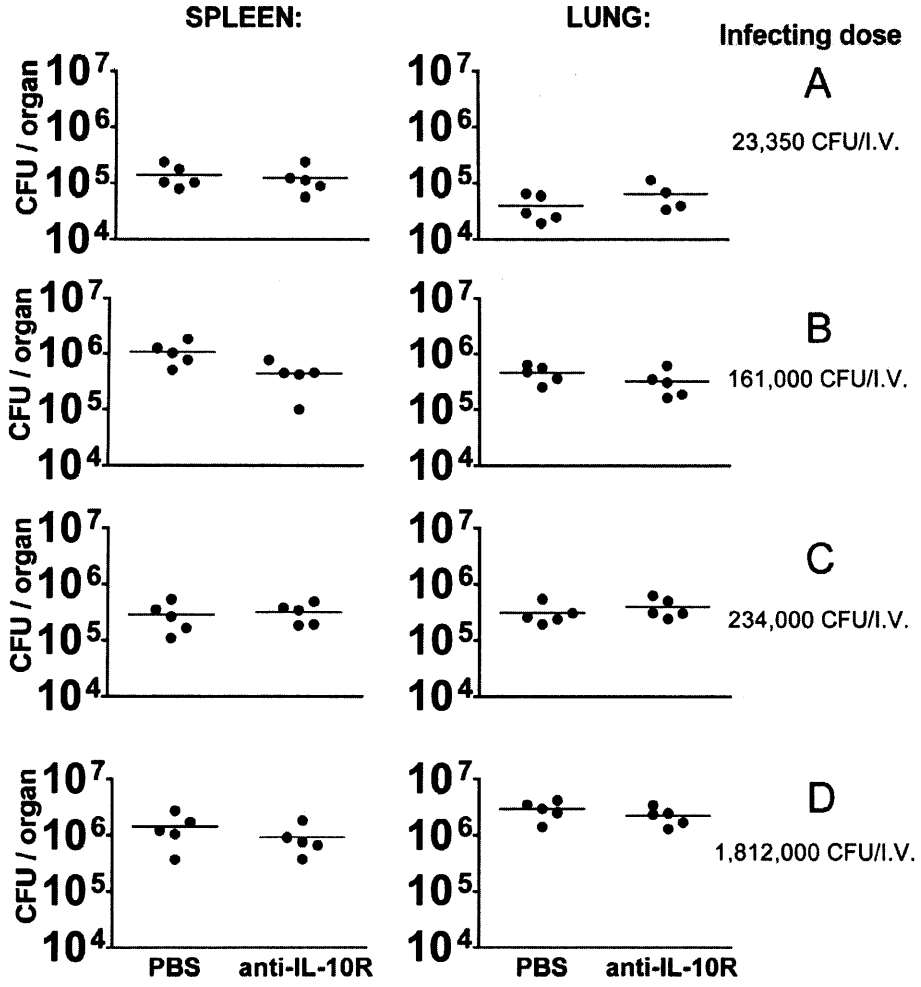


Figure 3.7: The effect of IL-10R neutralisation in vivo late after infection with H37Rv LSHTM is not dependent on initial infection dose.

BALB/c mice were infected via the I.V. route with either: (A) 23,350 CFU, (B) 161,000 CFU, (C) 234,000 CFU or (D) 1,812,000 CFU H37Rv LSHTM. At day 35 post infection mice were injected weekly I.P. with either anti-IL-10R mAb (1B1.3A; 0.5mg/mouse) or PBS (control). At day 77, mice were killed and bacterial burdens were determined in the spleen and lung as described in Materials and Methods (number of mice per group = 5). Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means. The effect of IL-10R mAb treatment was tested statistically using an unpaired Students *t* test (*, $P < 0.05$).

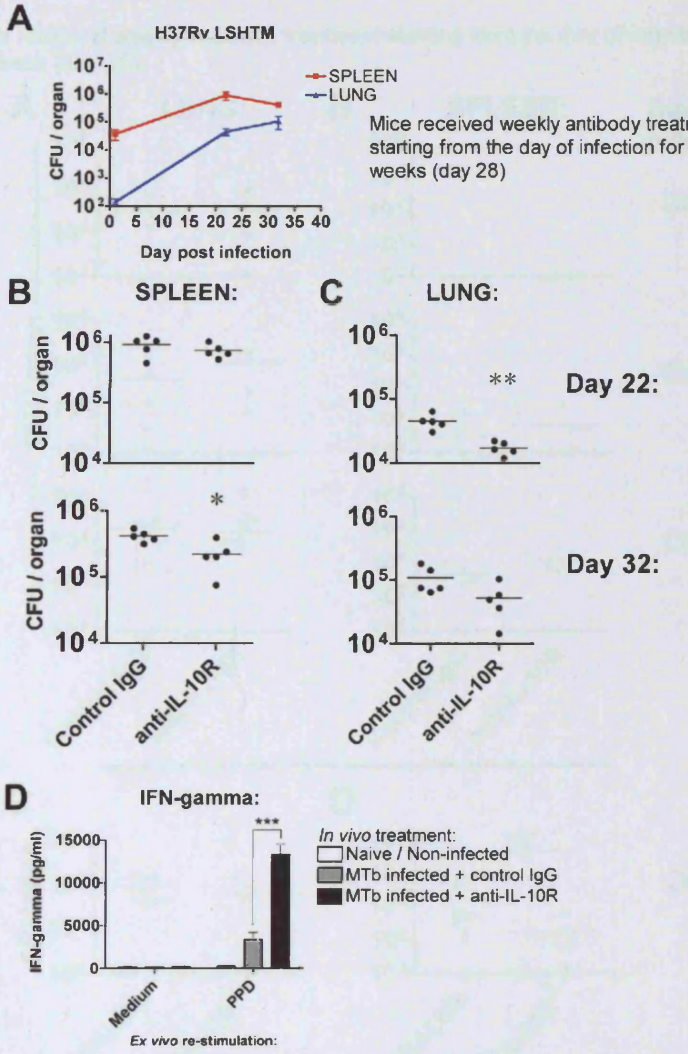


Figure 3.8: IL-10R neutralisation in vivo has a transient effect on bacterial burdens during early I.V. MTb infection with H37Rv LSHTM.

(A) Female BALB/c mice were infected I.V. with $2.2x10^5$ CFU H37Rv LSHTM strain and bacterial burdens were monitored in the spleen and lungs throughout the time course. From day 0 of infection mice were injected once weekly I.P. for 4 weeks with anti-IL-10R mAb (1B1.3A; 0.5mg/mouse) or control IgG (GL113). At days 22 and 32 post infection mice were killed and bacterial burdens were determined in the **(B)** spleen and **(C)** lung, as described in Materials and Methods (number of mice per group = 5). Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means. On Day 32, whole splenocytes were pooled from anti-IL-10R mAb or control IgG (GL113) treated mice and re-stimulated *ex vivo* with PPD (10μg/ml) alone or in the presence of anti-IL-10R mAb (10μg/ml). Supernatants were collected after 72 hours post stimulation and assayed by ELISA for **(D)** IFN-gamma production. The effect of IL-10R mAb treatment was tested statistically using an unpaired Students *t* test **(A-C)** or by use of a One-Way ANOVA with a Bonferroni multiple comparison post-test **(D)** (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$). Results shown are representative of two independent experiments.

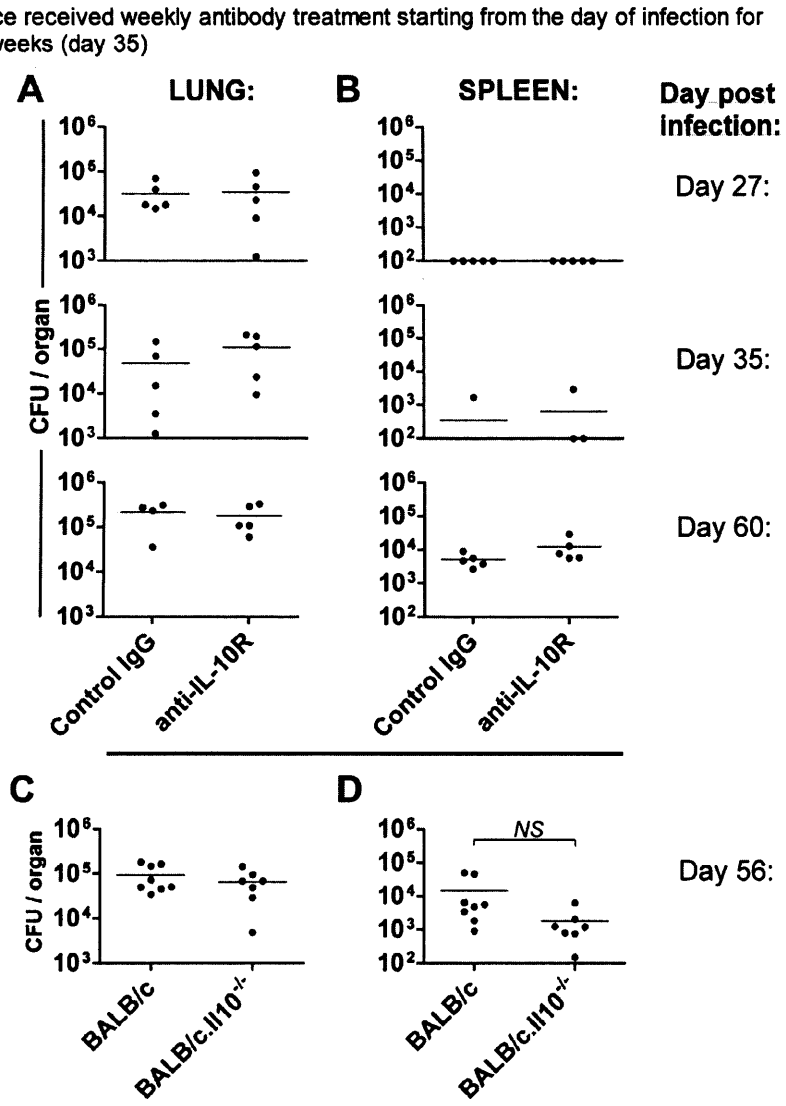


Figure 3.9: Bacterial load following early aerosol MTb infection is only minimally decreased in the absence of IL-10 signalling.

Female BALB/c mice were infected via the aerosol route with approximately 100 CFU H37Rv LSHTM strain. The bacterial burdens were monitored in the spleen and lungs throughout the time course. From day 0 of infection mice were injected once weekly I.P. for 5 weeks (until day 35), with either anti-IL-10R mAb (0.5mg/mouse) or control IgG (GL113). At days 27, 35 and 60 post infection mice were killed and bacterial burdens were determined in the lung (A) and spleen (B), as described in Materials and Methods (number of mice per group = 5). Alternatively BALB/c and BALB/c.*Il10*$^{-/-}$ mice were infected via the aerosol route and bacterial burdens were determined at day 56 post infection in the lung (C) and spleen (D) (number of mice per group = 7/8). Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means. The effect attributed to the absence of IL-10 was tested statistically using an unpaired Students *t* test (*NS* = non-significant).

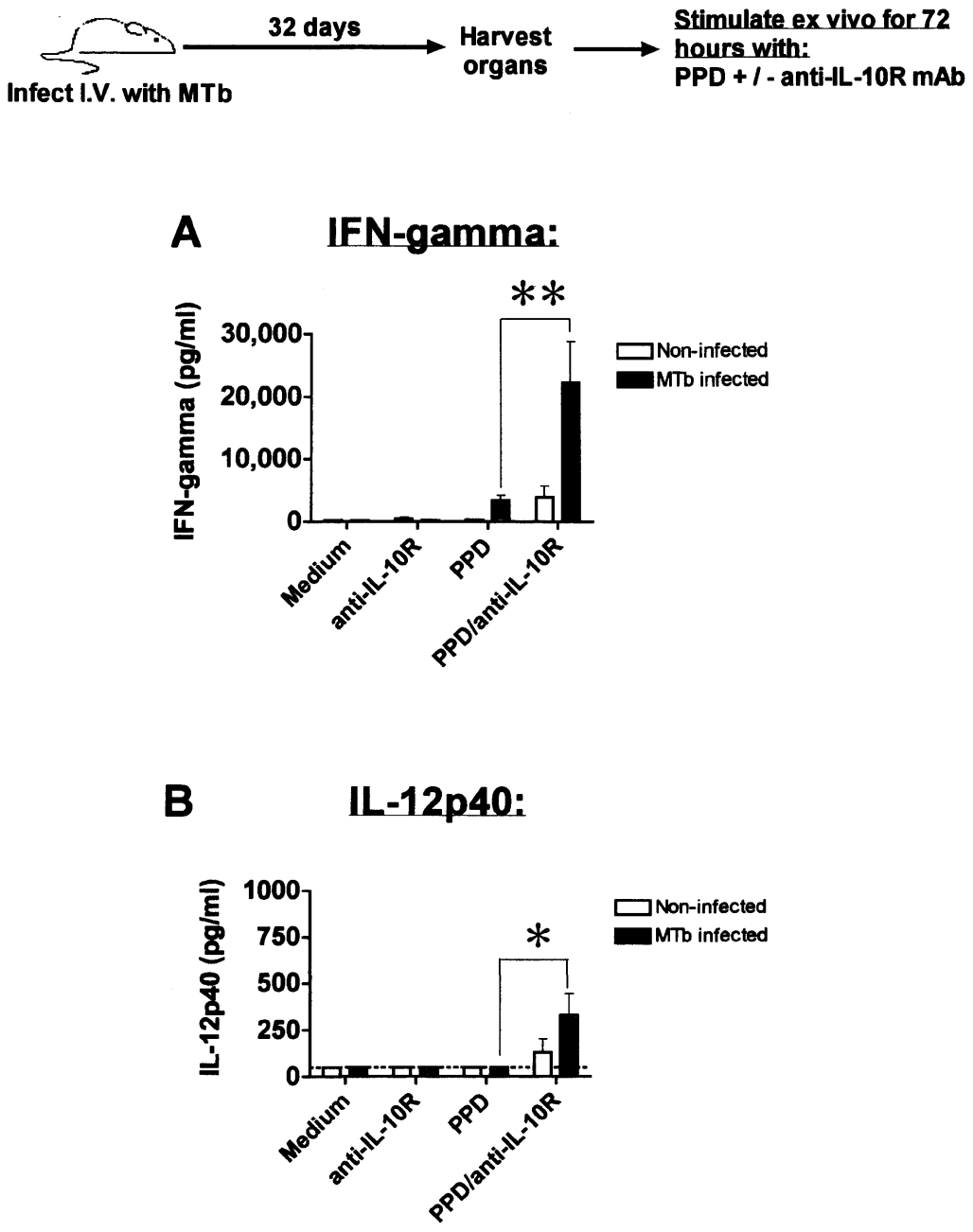


***Figure 3.10**: Neutralisation of endogenous IL-10 in ex vivo cultures from MTb infected mice enhances IFN-gamma responses.*

Whole splenocytes were pooled from either naïve or MTb infected mice (infected I.V. for 32 days with H37Rv LSHTM). Splenocytes were re-stimulated *ex vivo* with either, PPD (10μg/ml) alone or in the presence of anti-IL-10R mAb (both 10μg/ml). Supernatants were collected after 72 hours post stimulation and assayed by ELISA for (A) IFN-gamma or (B) IL-12p40 production. The effect of neutralising IL-10 signalling was tested statistically using an unpaired Students *t* test (*, $P < 0.05$; **, $P < 0.01$). Results shown are representative of two independent experiments.

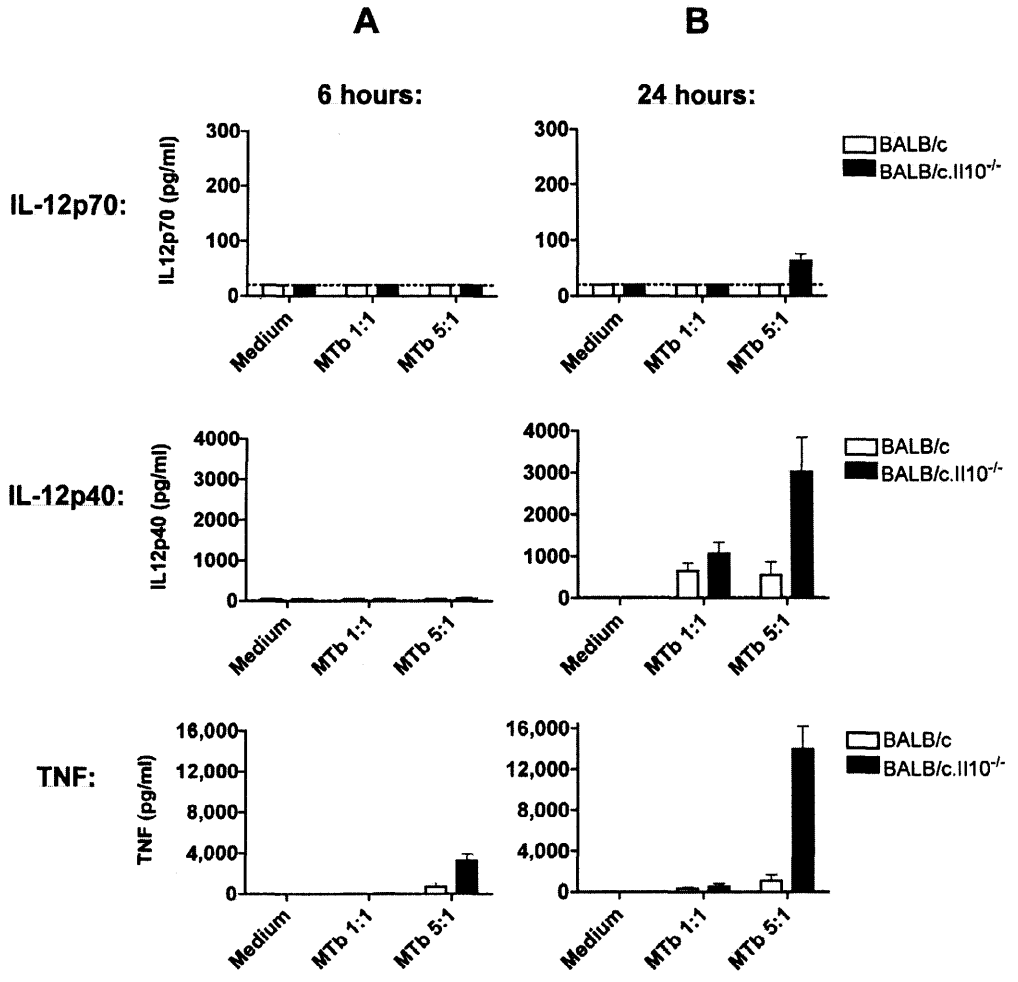


Figure 3.11: Endogenous IL-10 limits macrophages pro-inflammatory cytokine responses to MTb infection.

Macrophages cultured from the BM of wild-type BALB/c and BALB/c.*Il10*$^{-/-}$ mice and infected with MTb H37Rv LSHTM at an MOI of 1:1 and 5:1 (MTb:Macrophage). Supernatants were collected after (A) 6 hours and (B) 24 hours then assayed by ELISA for the presence of IL-12p70, IL-12p40 and TNF, as described in Materials and Methods.

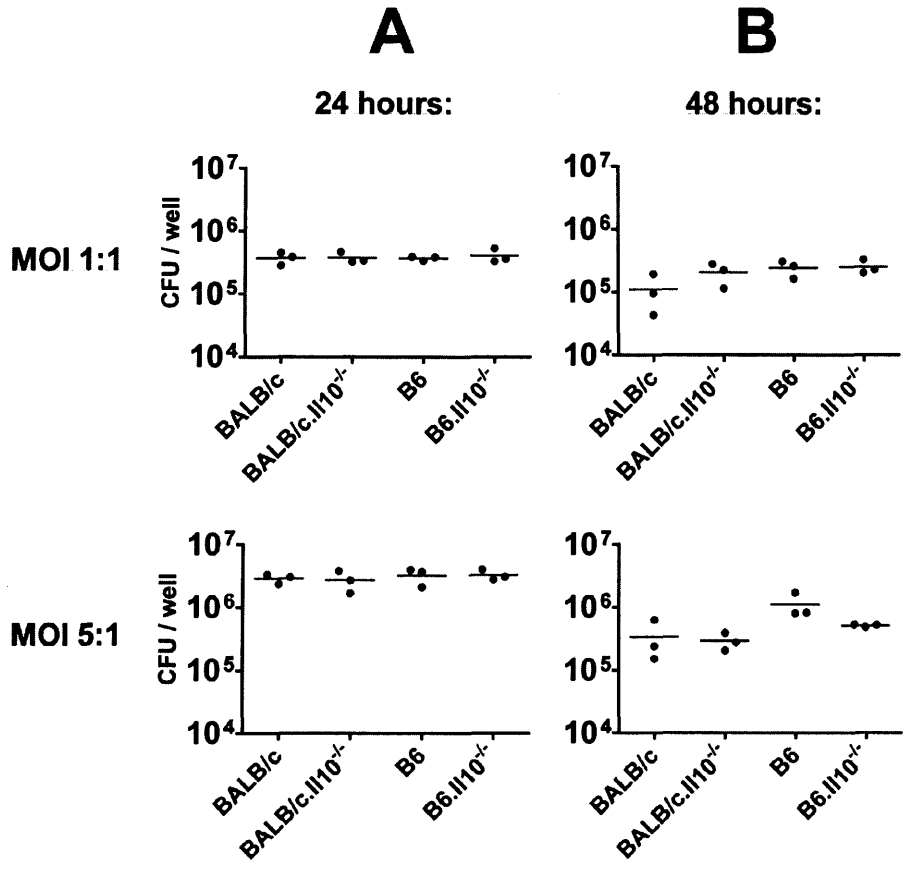


Figure 3.12: Despite the increase in pro-inflammatory cytokines in the absence of endogenous IL-10, the viability of MTb within BM macrophages is not altered.

Macrophages were cultured from the BM of wild-type and *Il10*$^{-/-}$ mice and infected with MTb H37Rv LSHTM at an MOI of 1:1 and 5:1 (MTb:Macrophage). After **(A)** 24 hours and **(B)** 48 hours, cells were harvested and CFU performed as described in Materials and Methods. Each point represents the CFU value from one tissue culture well with the horizontal lines representing the geometric means.

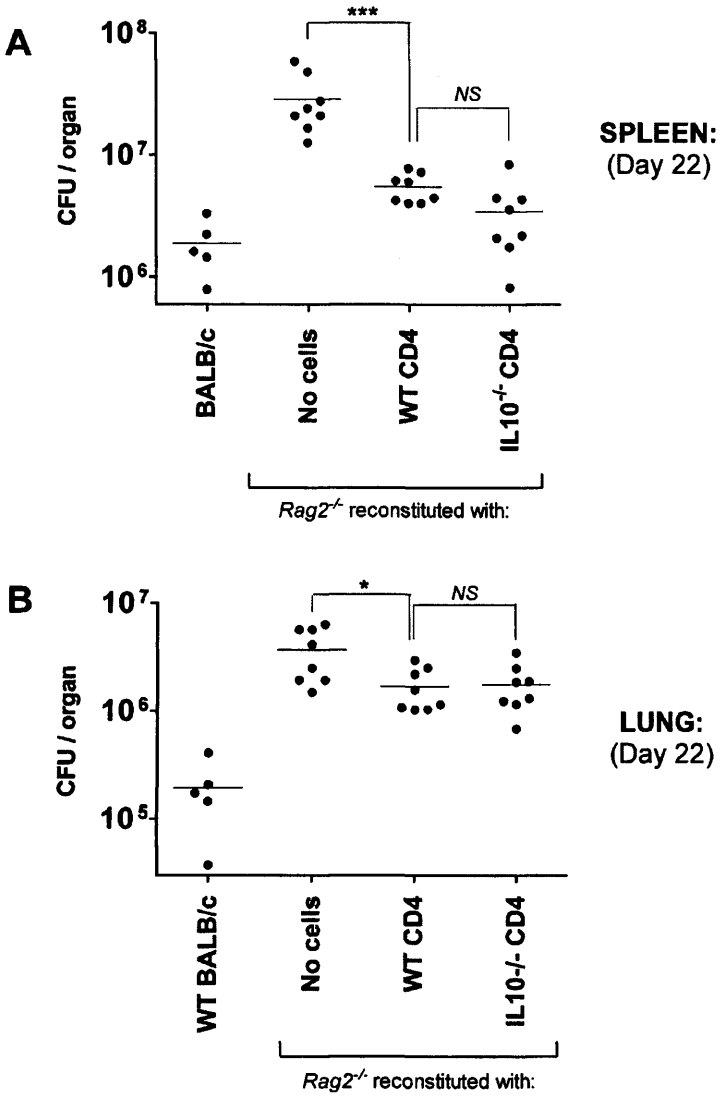


Figure 3.13: The protection afforded by CD4+ T cells in a transfer model of infection is not regulated by IL-10.

CD4+CD25- T cells were purified from the spleens of naïve WT BALB/c or BALB/c.*Il10*$^{-/-}$ mice as described in Materials and Methods. BALB/c.*Rag2*$^{-/-}$ mice were reconstituted I.V. the day before infection with $4x10^5$: *i)* no cells (PBS), *ii)* WT CD4+, or *iii) IL10*$^{-/-}$ CD4+. Mice were infected I.V. with approximately $1x10^5$ CFU H37Rv LSHTM. At day 22 post infection mice were killed and CFU were determined in the spleen **(A)** and lung **(B)**, as described in Materials and Methods (number of mice per group = 8). The effect of IL-10 was tested statistically using a One-Way ANOVA combined with a Bonferroni multiple comparison post-test (*, P <0.05; *** P <0.001; *NS* = non-significant). Results shown are representative of two independent experiments.

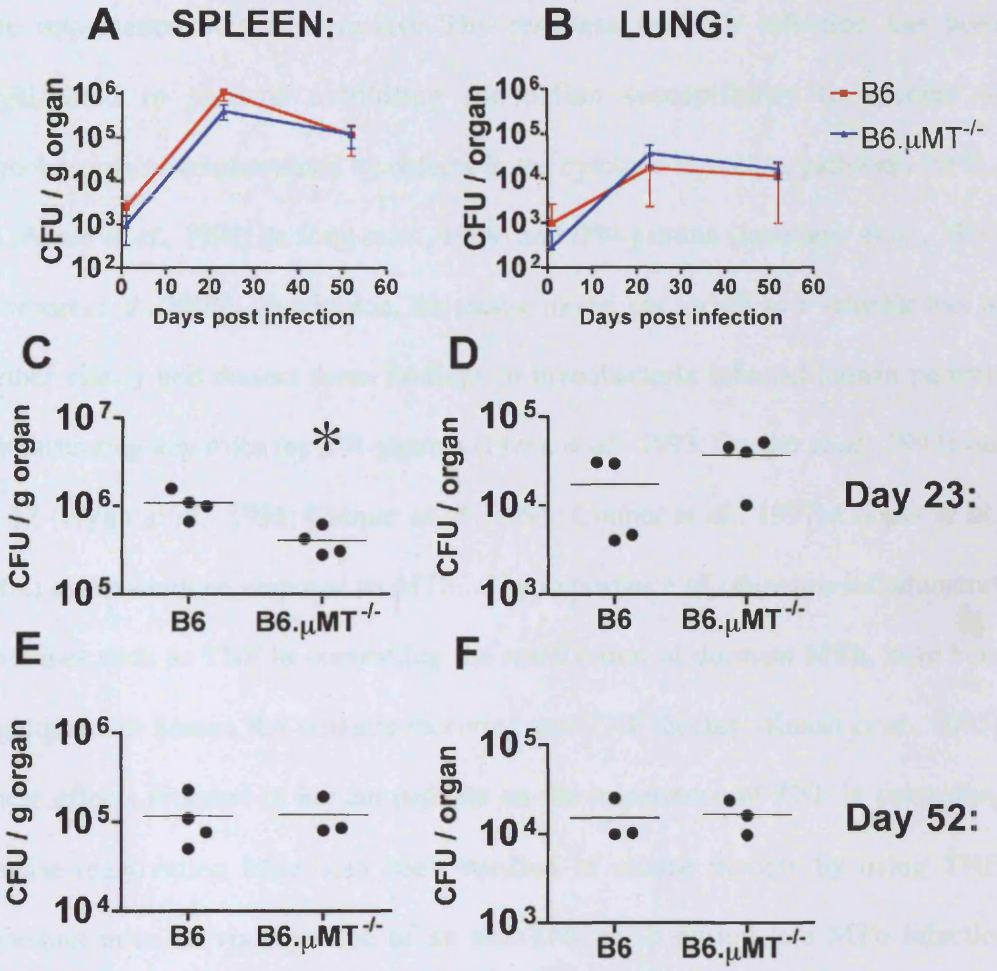


***Figure 3.14**: B6.μMT$^{-/-}$ mice show a transient decrease in CFU during the early phase of I.V. infection with H37Rv LSHTM.*

B6 and B6.*μMT*$^{-/-}$ mice were infected I.V. with 5.4x10^4 CFU H37Rv LSHTM strain. During the course of infection bacterial growth was determined in the spleen **(A)** and lungs **(B)**. At days 23 **(C)/(D)** and 52 **(E)/(F)** post infection mice were killed as described in Materials and Methods and bacterial burdens were determined. Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means. The effect on bacteraemia attributed to the absence of B cells was tested statistically using an unpaired Students t test (*, P <0.05).

3.9. Discussion:

The importance of the protective Th1 response to MTb infection has been highlighted in patients exhibiting Mendelian susceptibility to species of mycobacteria as demonstrated by defects in the cytokine signalling pathways for IL-12 (Altare *et al.*, 1998; de Jong *et al.*, 1998) and IFN-gamma (Jouanguy *et al.*, 1996; Newport *et al.*, 2003). In addition, the mouse model has served as a valuable tool to further clarify and dissect these findings in mycobacteria infected human patients demonstrating key roles for IFN-gamma (Flynn *et al.*, 1993; Cooper *et al.*, 1993) and IL-12 (Flynn *et al.*, 1995; Cooper *et al.*, 1995; Cooper *et al.*, 1997; Cooper *et al.*, 2002) in the immune response to MTb. The importance of other pro-inflammatory cytokines such as TNF in controlling the reactivation of dormant MTb, have been highlighted in human RA patients receiving anti-TNF therapy (Keane *et al.*, 2003). These effects reported in human patients on the importance of TNF in controlling disease reactivation have also been verified in mouse models by using TNF-knockout mice or via injection of an anti-TNF mAb during late MTb infection (Flynn *et al.*, 1995; Bean *et al.*, 1999).

The role of immuno-regulatory factors such as IL-10 in limiting pathogen clearance and thus promoting MTb survival in the mouse has been reported but the findings are controversial. During early MTb infection North *et al.*, (1998) and Jung *et al.*, (2003) have reported that in the absence of IL-10 there is no effect on bacterial burdens compared to wild-type mice. In contrast, Roach *et al.*, (2001b) have reported that in the absence of IL-10 there is a transient decrease in the bacterial level in the first 4 weeks post infection. Turner *et al.*, (2002) using an IL-10

transgenic mouse model, have shown that over-expression of IL-10 enhances bacterial burdens during late but not early MTb infection. Human studies using chronically infected TB patients have suggested a regulatory role for IL-10 in limiting IFN-gamma responses (Gong *et al.*, 1996) and proliferative responses (Boussiotis *et al.*, 2000) to the mycobacterial antigen PPD. Furthermore, Gerosa *et al.*, (1999) have reported the presence of CD4+ T cells in the BAL of active TB patients that produce both IFN-gamma and IL-10. Taken together these findings suggest that IL-10 affects bacterial clearance leading to chronic disease, however, this can only be inferred from studies in human at this stage.

Virulence between H37Rv strains:

Our data indicates a difference between the two H37Rv strains that could be linked to virulence. When intervening with an anti-IL-10R mAb late after infection H37Rv NIMR but not H37Rv LSHTM, a regulatory role for IL-10 on bacterial clearance was revealed. The lack of effect observed when neutralising IL-10 during infection with H37Rv LSHTM, was not dependent on bacterial load as mice infected with various infection doses of MTb before anti-IL-10R mAb did not clear bacteria more effectively than MTb infected control treated mice. When addressing the virulence between H37Rv strains by infection of immuno-deficient mice, studies in the laboratory have revealed a vast difference in virulence, as mice infected with H37Rv LSHTM and H37Rv NIMR had a mean survival time of 25 days and 46 days respectively. Finally, the *in vivo* growth curves for H37Rv LSHTM but not H37Rv NIMR were more reflective of those broadly reported in the literature. For these reasons we focussed our subsequent studies solely on H37Rv LSHTM.

A role for IL-10 during early H37Rv LSHTM infection:

We demonstrated that the timing of anti-IL-10R mAb treatment during MTb infection influenced whether an effect was seen on bacterial clearance. When infecting I.V. with H37Rv LSHTM we found an early role for IL-10 as treatment with anti-IL-10R gave a transient decrease in bacterial burdens in the lung at day 22 and spleen at day 32. When addressing a role for IL-10 in an aerosol infection model during early MTb infection, we saw no effect on bacterial burdens during anti-IL-10R mAb treatment. However, infection of BALB/c.*Il10*$^{-/-}$ mice showed a slight decrease in bacterial load in the spleen that was is in keeping with the findings by Roach *et al.*, (2001b), but failed to reach statistical significance. The slight difference in bacterial burdens observed between *Il10*$^{-/-}$ mice and mice that were treated with anti-IL-10R mAb could be due to *a)* the amount and timing of antibody treatment, *b)* the efficiency of antibody receptor blocking, *c)* the ability of the antibody to counteract an infection starting in the lungs or *d)* during the latter stages of mAb treatment mice may have elicited an anti-antibody response therefore neutralising any potential therapeutic effect. Following infection of *Il10*$^{-/-}$ mice we observed no effect on bacterial clearance in the lung, at day 56 post infection. This may result from a transient effect on bacterial load as demonstrated by Roach *et al.*, (2001b) whereby a 1-Log_{10} decrease in lung bacterial burdens was evident at day 28 but had diminished by day 42 post infection.

The role of IL-10 in during late infection with H37Rv LSHTM:

Since all the studies to date neutralising IL-10 during MTb infection were performed in *Il10*$^{-/-}$mice it has not been possible to determine a role for IL-10 during chronic infection. We sought to determine a role for IL-10 late after infection with H37Rv

LSHTM using an antibody to block the IL-10 receptor as studies by Turner *et al.*, (2002) using an IL-10 transgenic mouse under the control of the IL-2 promoter had identified a suppressive role for IL-10 late during MTb infection. Similarly, human studies have suggested that IL-10 suppresses protective immune responses leading to chronic infection (Gong *et al.*, 1996, Boussiotis *et al.*, 2000).

When mice were infected via either the I.V. or aerosol routes with H37Rv LSHTM and treated with anti-IL-10R mAb at a late stage after infection we did not observe a regulatory role for IL-10 on bacterial clearance. This finding suggested that IL-10 may not regulate bacterial clearance at this stage of infection. However, IL-10 may be playing a role but the level of neutralising mAb may not be sufficient to reverse the high level of infection. To address this possibility we infected mice with different doses of MTb before anti-IL-10R mAb was started. We still observed no effect on bacterial clearance following anti-IL-10R mAb treatment at any infection dose. With this in mind two further approaches could also be adopted to address the role of IL-10 during chronic infection a) if possible an IL-10 inducible knockout where IL-10 could be ablated *in vivo* post infection, and b) chemotherapy at the peak of MTb infection in order to lower the bacterial load before antibody treatment is initiated.

Enhanced IFN-gamma production in ex vivo re-stimulations in the absence of endogenous IL-10: new insights into interpreting human MTb studies.

In human studies it has been shown that neutralisation of IL-10 in PPD stimulated PBMC cultures from active TB patients restores IFN-gamma production (Gong *et al.*, 1996) or proliferative responses (Boussiotis *et al.*, 2000). The authors

interpreted the data suggesting that IL-10 must be regulating the immune response to MTb and therefore limiting pathogen clearance. Our data in a mouse model of MTb infection compliments the findings in the human studies, as stimulation of cells from MTb infected mice with PPD *in vitro* in the presence of anti-IL-10R mAb enhanced production of IFN-gamma and IL-12p40 (Fig. 3.10). This demonstrates that IL-10 is present although anti-IL-10R mAb treatment of mice late after infection with H37Rv LSHTM had not effect on bacterial clearance at this stage of infection (Fig. 3.5). As discussed earlier, demonstrating a role for IL-10 in regulating bacterial clearance at this late stage of infection may be difficult to achieve at high bacterial loads. Our findings at this stage of infection may also reflect that IL-10 may only regulate cytokine production and not bacterial killing as suggested by human studies. Furthermore, it is necessary to improve *ex vivo* assays in human studies in order to assess the effect of immune-modulators e.g. anti-IL-10R mAb, on bacterial killing. To compliment these human studies, experimental assays in the mouse also need to be modified to address subtler effects mediated by immune modulators.

IL-10 negatively regulates macrophage responses to in vitro MTb infection:

The data in human studies suggests that IL-10 functions to limit bacterial clearance as neutralisation of IL-10 in *ex vivo* PPD re-stimulations restores IFN-gamma responses (Gong *et al.*, 1996). In order to address a role for IL-10 on MTb killing we utilised a reductionist approach by infecting BM macrophages *in vitro* in absence of IL-10 to determine *a)* the induction of pro-inflammatory cytokines and *b)* macrophage MTb killing *in vitro*. Despite the finding that pro-inflammatory cytokine production from *Il10*$^{-/-}$ macrophages was enhanced following different burdens of infection *in vitro*, there was no enhancement of macrophage MTb killing.

The observation that MTb killing *in vitro* was not enhanced in the absence of IL-10, may be due to an over simplified *in vitro* system where T cells and NK cells were not present. Alternatively, MTb could be functioning to inhibit anti-microbial killing via inhibition of other signalling pathways. We are currently re-evaluating the role of IL-10 on macrophage activation by adding IFN-gamma to macrophage infection cultures to address this.

The source of IL-10 during MTb infection:

We sought to isolate a cellular source for IL-10 during MTb infection. In contrast to the published human studies by Gerosa *et al.*, (1999) and Boussiotis *et al.*, (2001), using adoptive transfer of CD4+.*Il10*$^{-/-}$ T cells into immuno-compromised *Rag2*$^{-/-}$ recipients and intracellular flow cytometry for detecting IL-10, we failed to confirm CD4+ T cells as a source for IL-10 at this stage during MTb infection, possibly reflecting the low frequency of IL-10 producing antigen specific T cells. The inability to detect IL-10 could be due to the low number of antigen specific T cells in the organs investigated, the sensitivity of the antibody or the kinetics or cellular origin of IL-10 cytokine production, all are key aspects that need to be more rigorously dissected. Besides CD4+ T cells, other cells that are involved in the innate and adaptive immune response have been shown to make IL-10 (Moore *et al.*, 2001).

With this in mind we addressed the role of B cells as a source of IL-10 by using B6.$\mu MT^{-/-}$. Following I.V. infection we could only address a transient role for B cells during MTb infection. Our studies compliment the findings of Bosio *et al.*, (2000) who also showed a transient role for B cells in limiting bacterial clearance during the

early immune response to MTb CDC1551. However, our data is in contrast to that published by Vordermeier *et al.*, (1996) who show an increase in bacterial burdens in the absence of B cells following I.V. MTb infection. The key differences that may explain the variability between these studies are the route of infection used, the time points examined and the strain of MTb, i.e. H37Rv or CDC1551. In addition, we are currently addressing the role of B cell derived IL-10 on MTb bacterial clearance in adoptive transfer studies using B cells from *Il10*$^{-/-}$ donors. Thus far CD4+ T cells and B cells do not appear to serve as a major source for IL-10 during the early immune response to MTb.

However, the potential for other cell types producing IL-10 also needs investigating as reports have demonstrated that both DC and macrophages readily produce IL-10 following TLR stimulation (Dillon *et al.*, 2004, Boonstra *et al.*, 2006) or via ligands that trigger non-TLR such as through DC-SIGN (Gagliardi *et al.*, 2005) or Dectin-1 (Rogers *et al.*, 2005). Interestingly, it has also been shown that regulation of the innate immune response to Listeria infection is by IL-10 derived from a population of CD11b+ cells (Carrero *et al.*, 2006).

Chapter 4: The Potential Role for Naturally Occurring Regulatory T cells As Mediators of MTb Survival and Persistence.

4.1. The potential role for naturally occurring regulatory T cells as mediators of MTb survival and persistence, aims of the investigation:

To address the role of regulatory T cells in MTb we sought to:

1) Determine the kinetics of Tregs in the lung following aerosol MTb infection.

2) Identify whether the immune response and moreover bacterial clearance, is altered in mice depleted of Tregs prior to infection?

3) Evaluate the immunosuppressive role of TGF-beta during MTb infection.

4) Using an adoptive transfer model:

a) Can Tregs limit the immune response following MTb infection?

b) Do Tregs mediate suppression by IL-10 dependent mechanisms?

4.2. Background:

Naturally occurring CD4+CD25+ Tregs were originally described by Sakaguchi *et al.*, (1995) as essential regulators of autoimmunity. More recently, Tregs have been shown to play regulatory roles in allergy (Kearley *et al.*, 2005) and infection with agents such as *Listeria* (Kursar *et al.*, 2002), *Plasmodium* (Hisaeda *et al.*, 2004) HSV (Suvas *et al.*, 2003) and *Leishmania* (Belkaid *et al.*, 2002; Mendez *et al.*, 2004). In addition to the marker CD25 (IL-2R-alpha), Tregs also express high levels of CTLA-4, GITR, CD103 and OX40 (Fontenot *et al.*, 2005b). Interestingly like CD25, all these markers are up regulated on all T cells following activation, thus making CD25 in the context of infection, an unreliable marker for defining Tregs (Hawrylowicz, 2005). However, the discovery of the Treg lineage specific transcription factor FoxP3 has further facilitated studies on Tregs.

The role of FoxP3+ Tregs during MTb infection has only now been recently reported. Recent studies of PBMC from human TB patients have identified an increase in FoxP3 mRNA expression (Guyot-Revol *et al.*, 2006) and by flow cytometry, Ribeiro-Rodrigues *et al.*, (2006) have reported an increase in the percentage of CD4+CD25+FoxP3+ Tregs that have suppressive activity *in vitro*. In the mouse model, there is limited data on the role of Tregs during MTb infection. Quinn *et al.*, (2006) have shown by flow cytometry that following intranasal infection with BCG there is an increase in the total number of CD4+CD25+FoxP3+ Tregs. In a model for depletion of Tregs by treating mice with the anti-CD25 mAb (clone PC61), they showed no effect on bacterial burdens or histology, but did show that IFN-gamma production by CD4+ T cells was enhanced. In the same study, Quinn *et al.*, (2006) also presented a limited amount of data demonstrating that depletion of Tregs via treatment of mice with anti-CD25 mAb had no effect on clearance of MTb. However, it is well documented that *in vivo* treatment of mice with anti-CD25 mAb depletes a subset of naturally occurring CD4+CD25+ Tregs. One caveat of this approach is that anti-CD25 mAb will also deplete recently activated effector T cells that also express CD25, thus numerous studies in the field of Tregs utilise transfer models. To address a role for Tregs during murine MTb infection, we adopted a three pronged approach utilising *i)* flow cytometry to track FoxP3 expression; *ii)* antibody depletion of CD25+ cells; *iii)* adoptive transfer of specific T cell populations into immuno-compromised recipients.

4.3. CD4+FoxP3+ Tregs increase at the site of MTb infection following aerosol infection:

Although an increase in FoxP3 expression by mRNA expression and flow cytometry in the blood of TB patients has been shown (Guyot-Revol *et al.*, 2006; Ribeiro-Rodrigues *et al.*, 2006), there is no published data on FoxP3 expression following MTb infection in mice. We therefore wanted to establish whether following aerosol infection there was an increase in Tregs at the site of infection, as identified by expression of the markers CD4, CD25 and FoxP3. However, before we could address this question and in order to optimise the system, we titrated the FoxP3 antibody (2μg/ml down to 0.125μg/ml) on whole splenocytes suspensions from naïve BALB/c mice. In addition, we also confirmed the specificity of the FoxP3 antibody by testing it on whole splenocytes obtained from *foxp3*$^{-/-}$ mice and as expected they were negative for FoxP3 compared to wild type controls.

To address the influx of Tregs during MTb infection, BALB/c mice were infected with H37Rv LSHTM strain via the aerosol route with approximately 100 CFU. During the course of infection mice were killed, cell suspensions from the lungs and spleen were prepared and stained for expression of CD4, CD25 and FoxP3 (Fig. 4.1A). By flow cytometry we observed a sustained increase in the percentage of lung CD4+ T cells from day 8 post infection compared to non-infected control mice (Fig.4.1B). The observed increase in CD4+ T cells was accompanied by an increase in the percentage CD4+CD25+FoxP3+ Tregs and CD4+CD25-FoxP3+ Tregs from day 8 (Fig. 4.1C). In addition, a subset of CD25^{low}Fox3- expressing T cells was evident early on during the time-course, which could have potentially constituted a

population of early effector T cells, but these failed to increase during the course of infection.

During the progression of infection (Fig. 4.2A), we observed an increase in the total numbers of CD4+FoxP3+ T cells in the lung from day 8 to day 29 post infection that was independent of the marker CD25 (Fig. 4.2C and D). This increase in CD4+CD25+FoxP3+ and CD4+CD25-FoxP3+ Tregs was in contrast to the numbers of effector T cells (CD4+CD25+FoxP3-), that remained unchanged until day 29 post infection and then increased until day 50 when the experiment was terminated (Fig. 4.2B), which is in keeping with reports by others (Junqueira-Kipnis *et al.*, 2004). In contrast, we observed no effect on the total numbers of either CD4+ T cell sub-population in the spleen following aerosol infection (data not shown).

4.4. The role of naturally occurring CD25+ Tregs during MTb infection:

4.4.1. Anti-CD25 mAb treatment of mice before MTb infection does not affect bacterial load but enhances IFN-gamma production:

As discussed previously, we observed an increase in lung CD4+CD25+FoxP3+ Tregs following aerosol exposure to MTb. To address a functional role for CD25+ Tregs as negative regulators of the immune response to early MTb, mice were treated on day -4, 2 and 0 before infection, with anti-CD25 mAb (PC61; 0.5mg/mouse) or control IgG (GL113) to specifically deplete naturally occurring CD25+ Tregs. On day 0 before infection the depletion of Tregs was confirmed by flow cytometry and was greater than 80% (Fig. 4.3). We adopted this early anti-CD25 mAb treatment schedule, as anti-CD25 mAb will also deplete recently

activated CD4+CD25+ effector T cells, since naïve CD4+ T cells up-regulate CD25 shortly after activation (Shevach, 2006). This approach has been widely used to address the role of Tregs in infections such as *Listeria* (Kursar *et al.*, 2002), *Plasmodium* (Hisaeda *et al.*, 2004), *Leishmania* (Mendez *et al.*, 2004) and more recently BCG (Quinn *et al.*, 2006).

In addition to CD25+ Tregs, we also sought to determine a role for TGF-beta during MTb infection. As discussed earlier, TGF-beta is a widely distributed immunosuppressive cytokine that is important in the down-regulation of Th1 and Th2 cell mediated immune responses (Letterio *et al.*, 1998). Studies in mice have reported that Treg derived TGF-beta plays a key role in regulating T cell mediated colitis (Powrie *et al.*, 1996, Maloy *et al.*, 2003) and is essential in the maintenance of the Treg population (Marie *et al.*, 2005). The role of TGF-beta during TB infection has not been well documented. In human studies it has been reported that TGF-beta is elevated in the serum of TB patients compared to healthy controls (Fiorenza *et al.*, 2004 and Guyot-Revol *et al.*, 2006). To address the roles of TGF-beta in MTb we used *in vivo* antibody neutralisation as it is well documented that *Tgf-B1*$^{-/-}$ mice die at 3-4 weeks of age due to an overwhelming lymphoproliferative disorder (Shull *et al.*, 1992).

BALB/c mice were treated with anti-CD25 mAb (0.5mg/mouse) at day -4, -2 and day 0 before infection as described above (Fig. 4.3). Similarly, as IL-10 and TGF-beta also function as immune-regulators, some mice were also administered with anti-IL-10R mAb (0.5mg/mouse), anti-TGF-beta antibody (1.5mg/mouse) or control IgG before I.V. H37Rv infection, then once weekly during the course of the

infection. On the day of infection, the efficiency of Treg depletion in mice that received anti-CD25 mAb as determined by flow cytometry for expression of CD4, CD25 and FoxP3 in the spleen was greater than 80% (Fig. 4.3). Following infection mice were killed at day 1 or 22 (Fig. 4.4) to determine the bacterial growth within mice, then at day 33 final bacterial burdens in the spleen (Fig. 4.4B) and lung (Fig. 4.4C) were determined. Treatment of mice with anti-CD25 mAb alone or anti-TGF-beta antibody alone had no effect on bacterial burdens during the early stage of infection. As demonstrated previously (chapter 3; Fig. 3.8), treatment of mice with anti-IL-10R mAb alone had a significant effect on bacterial burdens in the spleen at day 33 (Fig. 4.4.B). Strikingly, co-administration of both anti-CD25 and anti-IL-10R mAb's significantly enhanced bacterial clearance in the spleen (Fig. 4.4B) but not lung (Fig. 4.4C) compared to single treatment controls at the time-points investigated. The observed decrease in bacterial burdens following combined treatment of mice with both anti-CD25 mAb and anti-IL-10R mAb could not be further enhanced in the presence of anti-TGF-beta antibody (Fig. 4.4B and C). Our findings suggest only a minor effect when depleting CD25+ cells during early MTb infection except when administered in the presence of anti-IL-10R mAb.

In addition, we also examined the effect of anti-CD25 mAb treatment of mice during aerosol MTb infection (Fig. 4.5), however treatment of mice under the same regimen as described above, with anti-CD25 mAb alone or combined with anti-IL-10R mAb did not affect bacterial burdens in either the spleen (Fig. 4.5B) or lungs (Fig. 4.5C) at days 27, 35 or 60 post infection. Our findings illustrate that CD25+ Tregs do not have dominant role in regulating the early innate immune response to MTb as treatment with anti-CD25 mAb alone is not sufficient to enhance bacterial clearance.

We postulate that in the absence of CD25+ cells, other regulatory factors such as IL-10 can still regulate the immune response.

To address whether anti-CD25 mAb treatment enhanced T-cell responses during I.V. MTb infection, whole splenocytes from infected anti-CD25 mAb or control IgG treated mice were re-stimulated for 72 hours in the presence of PPD (Fig 4.6). Re-stimulation of whole splenocyte cell suspensions *ex vivo* with PPD gave an increase in production of IFN-gamma ($P < 0.01$; Fig 4.6A), as compared to infected control IgG treated mice. Similarly, IL-12p40 (Fig. 4.6B) and TNF (Fig 4.6C) were also enhanced in mice that were treated *in vivo* with anti-CD25 ($P < 0.05$), but this could only be observed when the re-stimulation was performed in the presence of IL-10 neutralising antibodies. Our data implies that the increase in IFN-gamma production in re-stimulations from anti-CD25 mAb alone treated mice is probably not sufficient to mediate bacterial clearance *in vivo*.

4.5. Immuno-compromised *Rag*$^{-/-}$ mice as a model for determining the role of Tregs during early MTb infection:

4.5.1. Determining the kinetics of MTb infection in Rag1$^{-/-}$ *mice:*

When addressing a role for CD4+CD25+Tregs during MTb infection using anti-CD25 mAb depletion we observed no effect on bacterial load. It is known that treatment of mice with anti-CD25 mAb not only depletes naturally occurring CD4+CD25+Tregs but can also deplete recently activated CD4+ effector T cells. In accordance with this, not all Tregs express the marker CD25, therefore a small subset of CD4+CD25-FoxP3+ Tregs - approximately 20-30%, cannot be depleted

and will persist with regulatory capacity. With this in mind we used an adoptive transfer model as a complimentary approach to address the role of CD4+CD25+ Tregs during early MTb infection. The *Rag*$^{-/-}$ adoptive transfer model has been widely used in determining the role of Tregs in murine models of colitis (Powrie *et al.*, 1996, Asseman *et al.*, 2003) and *Leishmania major* (Belkaid *et al.*, 2002; Mendez *et al.*, 2004).

We first sought to define the kinetics of I.V. MTb infection in immuno-compromised *Rag*$^{-/-}$ mice. Wild-type B6 and B6.*Rag1*$^{-/-}$ mice were infected I.V. with H37Rv LSHTM (Fig. 4.7A) with bacterial burdens being determined in the spleen (Fig. 4.7B) and lung (Fig. 4.7C) at the time points indicated. Following I.V. infection there was no difference in bacterial load between B6 and B6.*Rag1*$^{-/-}$ mice until day 14 post infection, where B6.*Rag1*$^{-/-}$ displayed a 10-fold higher bacterial load that was further increased by day 21 as the infection progressed (Fig. 4.7B). In the lung, the infection progressed more slowly with B6.*Rag1*$^{-/-}$ mice displaying an increased bacterial load at day 21 compared to B6 mice (Fig. 4.7C). For the subsequent adoptive transfer studies we chose day 21 as a suitable end-point for experiments due to the observed correlation between protection and bacterial load in both the spleen and lungs.

4.5.2. CD4+CD25+ Tregs have a limited effect on bacterial clearance:

Furthermore, to address the potential role of Tregs during MTb infection, BALB/c.*Rag2*$^{-/-}$ mice were reconstituted the day before infection with either naïve CD4+CD25- T cells alone or together with increasing numbers of naïve

CD4+CD25+ Tregs. The following day mice were infected I.V. with H37Rv LSHTM. Immuno-compromised BALB/c.*Rag2*$^{-/-}$ recipients (no cells) and BALB/c.*Rag2*$^{-/-}$ mice receiving CD4+CD25+ Tregs failed to control MTb infection with bacterial burdens significantly higher compared to wild-type BALB/c. Re-constitution of BALB/c.*Rag2*$^{-/-}$ with CD4+CD25- T cells conferred significant protection to infection with H37Rv in both the spleen (Fig. 4.8A) and lung (Fig. 4.8B). There was evidence to suggest that the presence of Treg at a non-physiological ratio of 1:1 (Naïve CD4+CD25- T cell: CD4+CD25+ Treg), limited the immune response to H37Rv infection in the spleen (Fig. 4.8A) and lungs (Fig. 4.8B), however this finding failed to reach statistical significance.

We have demonstrated that treatment of mice with anti-CD25 mAb alone has no effect on bacterial clearance unless it is administered together with anti-IL-10R mAb. We sought to verify our findings above using an adoptive transfer model using CD4+CD25- T cells from wild-type and *Il10*$^{-/-}$ mice. BALB/c.*Rag2*$^{-/-}$ mice were re-constituted with either whole CD4+ T cells (as reported previously in Chapter 3; Fig 3.13) or Treg depleted CD4+CD25- T cells from either wild-type or *Il10*$^{-/-}$ mice (Fig. 4.9). When CD4+CD25+ Tregs were removed from whole CD4+ T cell populations to yield CD4+CD25- T cells, there was no enhanced protective effect as compared to whole CD4+ T cells in either the spleen (Fig 4.9A), or the lungs (Fig. 4.9B). This observation is in accordance with our earlier findings, when depletion of CD25+ cells via mAb treatment had no effect on bacterial clearance suggesting that CD25+ Tregs play a minimal role in the immune response to MTb at this stage. The data suggests that CD4+ T cell derived IL-10 and more specifically

CD4+CD25+ Treg derived IL-10 plays only a limited role at this stage during early infection with H37Rv LSHTM.

4.5.3. Transfer of CD4+ CD25+ T cells from MTb infected donors mediates protection not suppression to subsequent MTb infection:

Since we did not observe any significant effect following reconstitution of mice with Tregs at non-physiological levels, we wished to determine based on our FACS analysis (Fig. 4.1), whether MTb expanded Tregs would be more effective in regulating the immune response to MTb. To address this we infected mice for 14 days before we attempted to enrich antigen specific Tregs, as at this time-point our FACS analysis revealed that the ratio of Tregs to effectors was significantly higher.

To address this BALB/c.*Rag2*$^{-/-}$ mice received either a) CD4+CD25- T cells from naïve donors or b) CD4+CD25+ T cells from MTb infected donors (from mice that had been infected for 14 days). Mice were then infected I.V. with H37Rv LSHTM for 22 days before bacterial burdens were determined in the spleen (Fig. 4.10A) and lungs (Fig. 4.10B), in comparison to infected wild-type BALB/c. As predicted, MTb infection of BALB/c.*Rag2*$^{-/-}$ recipients (no cells) or mice receiving CD4+CD25+ Tregs (from naïve donors) failed to control infection. However, transfer of CD4+CD25- T cells (from naïve mice) or CD4+CD25+ T cells (from MTb donors), conferred protection in the spleen (Fig. 4.10A) and lung (Fig. 4.10B). This effect was comparable to wild-type BALB/c as shown by a significant decrease in bacterial burdens. Our data demonstrates that in the first 14 days following I.V. infection CD4+CD25+ effector T cells are generated that mediate protection to infection with

MTb which may explain why anti-CD25 mAb treatment during *in vivo* infection was not effective.

4.6. Figures:

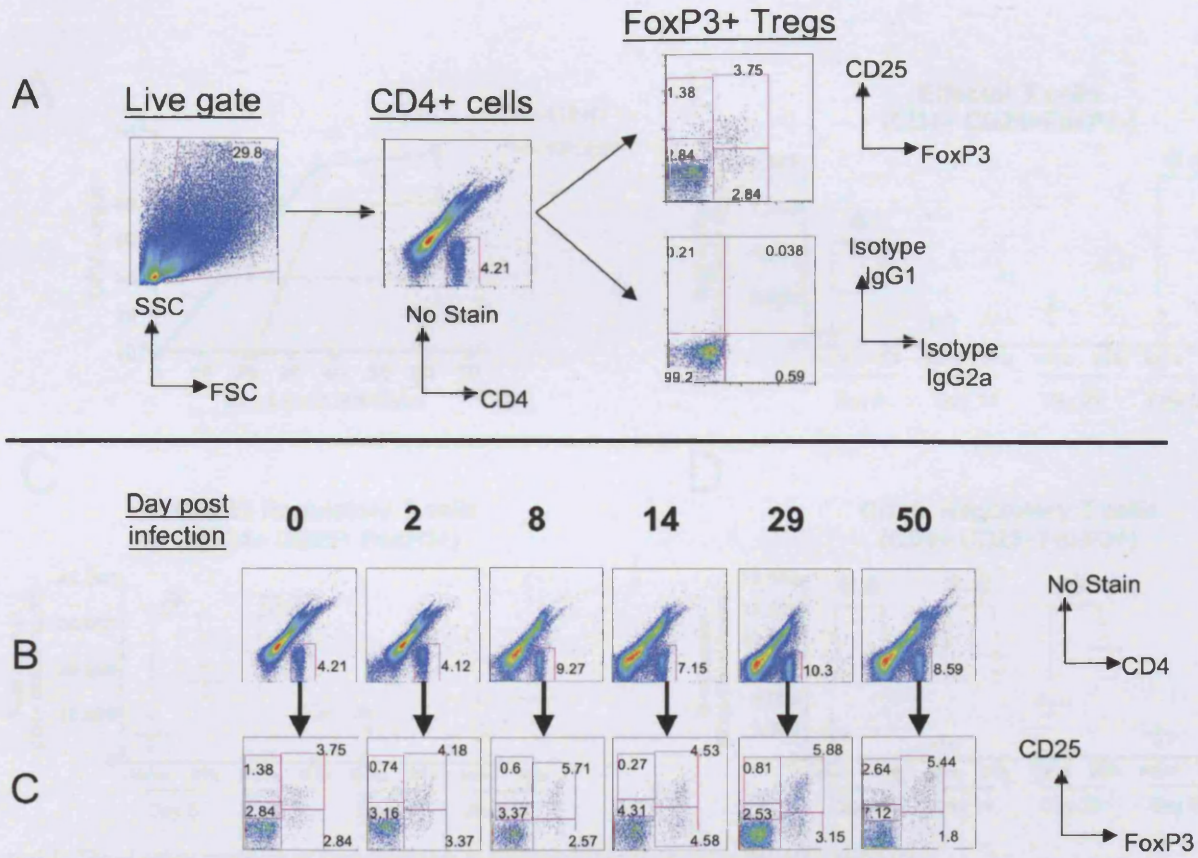


Figure 4.1: Regulatory T cells accumulate in the lungs of mice shortly after MTb infection.

BALB/c mice were infected with approximately 100 CFU H37Rv LSHTM strain via the aerosol route. At the time points indicated post infection the lungs were removed and stained for CD4, CD25 and FoxP3 expression as described in Materials and Methods. Viable cells were selected on their FSC / SSC profile **(A)** to define the CD4+ cells and CD4+FoxP3+ Tregs. At the time-points indicated, CD4+ cells **(B)** were selected then analysed for expression of CD25 and FoxP3 **(C)**. The percentage values indicated on the plots for CD4+ cells are of total live cells and the expression of CD25 and FoxP3 are shown as a percentage of total CD4+ T cells. Each plot is representative of 3 individual mice and the data shown is representative of 2 independent experiments

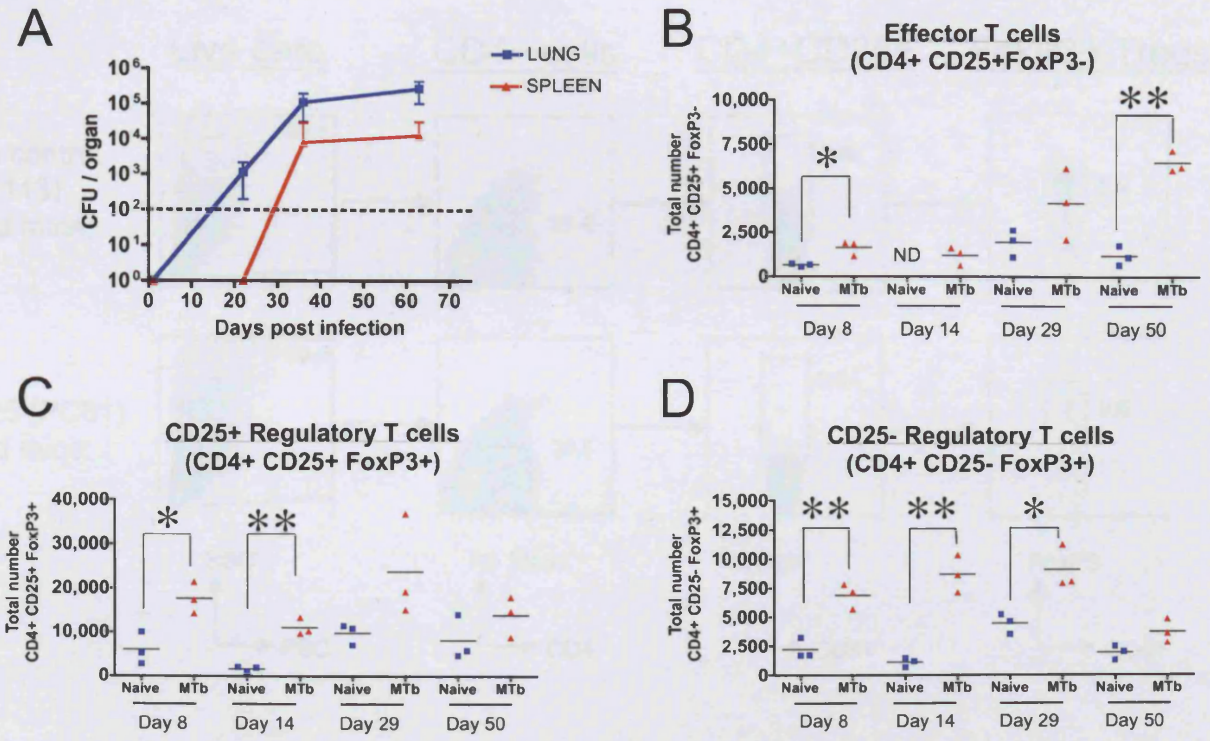


Figure 4.2: The absolute numbers of Tregs increase in the lungs of mice shortly after MTb infection.

BALB/c mice were infected with approximately 100 CFU H37Rv LSHTM strain via the aerosol route and the bacterial load was monitored during the course of infection (A). Total numbers of CD25+ effector (B), CD25+FoxP3+ Treg (C) and CD25-FoxP3+ Treg (D) during the infection time course are shown. Each data point represents the value obtained from one mouse lung with the horizontal lines representing the geometric means. The influx of Tregs during infection was tested statistically using a Students *t* test (*, $P < 0.05$; **, $P < 0.01$). The data shown is representative of 2 independent experiments.

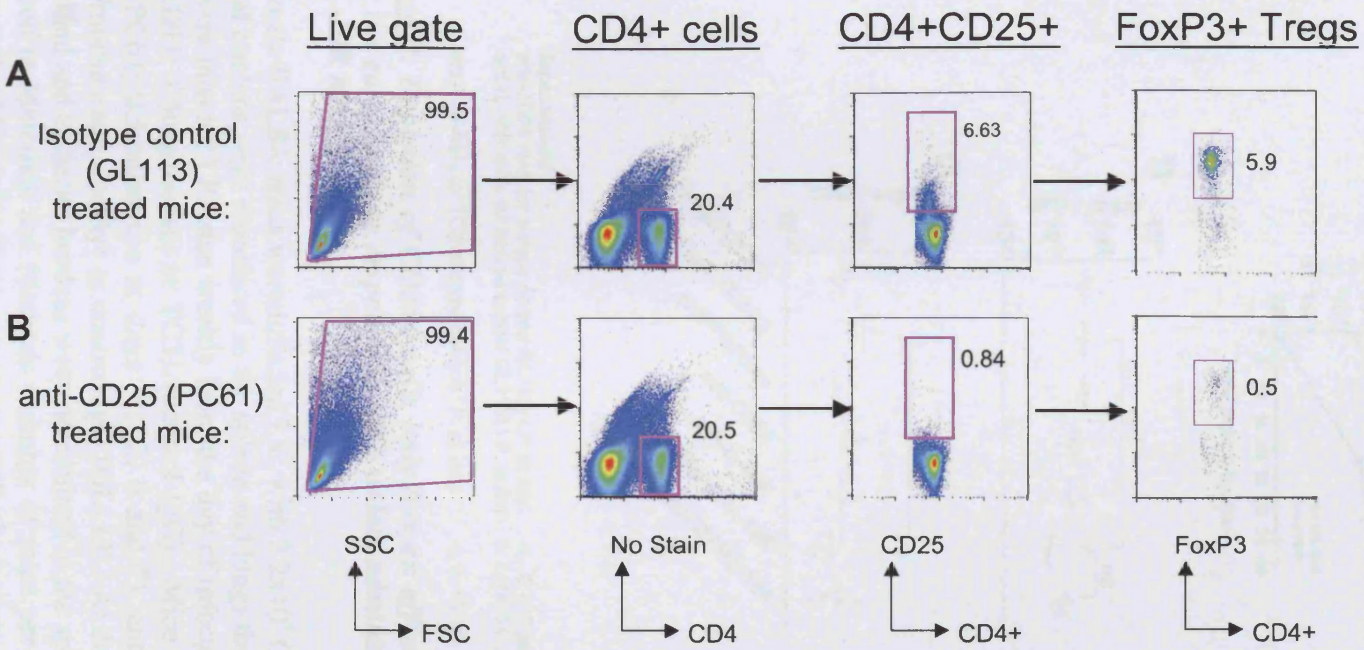
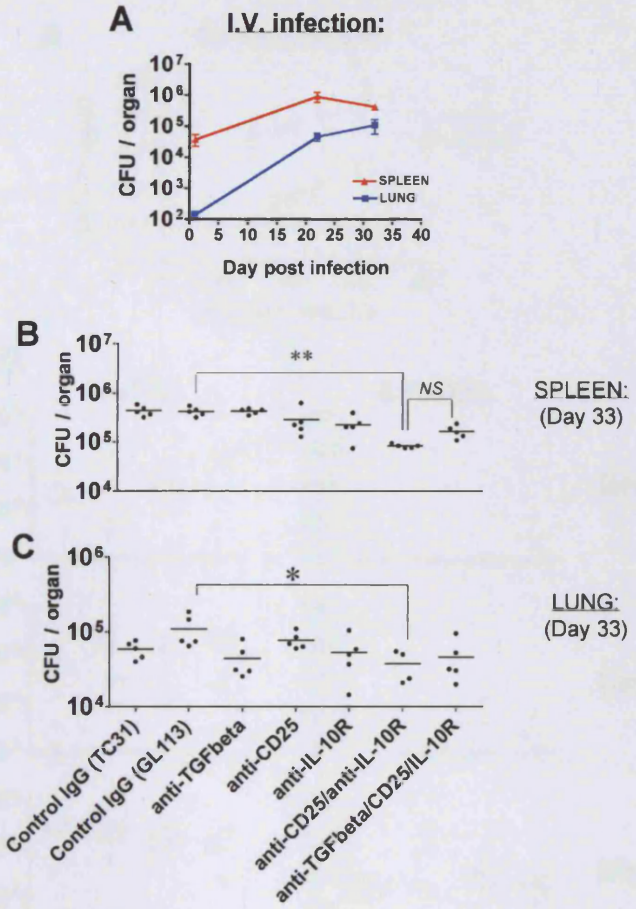


Figure 4.3: anti-CD25 mAb treatment of mice depletes naturally occurring CD4+CD25+FoxP3+ Tregs.

BALB/c mice were treated I.P. with either **(A)** control IgG (GL113) or **(B)** anti-CD25 (PC61; 0.5mg/mouse) on day -4, -2 and day 0 before being sacrificed. Whole splenocytes suspensions were prepared as described in Materials and Methods and stained for the presence of CD4+CD25+FoxP3+ Tregs. Data shown is representative of 2 independent experiments.



Mice received:
anti-CD25 mAb (or isotype control GL113) I.P. at day: -4, -2, 0, 7 post infection.
anti-IL-10R mAb (or isotype control GL113) I.P. at day: 0, 7, 14, 21, 28 post infection.

anti-TGF-beta (or TC31 isotype control) I.P. at: day: -1, 6, 13, 20, 27 post infection.

Figure 4.4: Depletion of CD25+ cells only has an effect on bacterial clearance during the early immune response to MTb unless administered in the presence of anti-IL-10R mAb.

(A) Female BALB/c mice were infected I.V. with $2.2x10^5$ CFU H37Rv LSHTM and bacterial burdens were monitored in the spleen and lungs throughout the time course. Mice were injected I.P. once weekly from the day of infection with either anti-TGF-beta (1D11; 1.5mg/mouse) or TC31 (control IgG). Mice also received anti-CD25 mAb (PC61; 0.5mg/mouse at days -4, -2, 0 and 7), anti-IL10R mAb (1B1.3A; 0.5mg/mouse once weekly) or control IgG (GL113). At day 33 post infection mice were killed and bacterial burdens were determined in the spleen **(B)** and lung **(C)**, as described in Materials and Methods (number of mice per group = 5). Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means. The effect of mAb treatment was tested statistically using a One-Way ANOVA combined with a Bonferroni multiple comparison post-test (*, P <0.05; **, P <0.01).

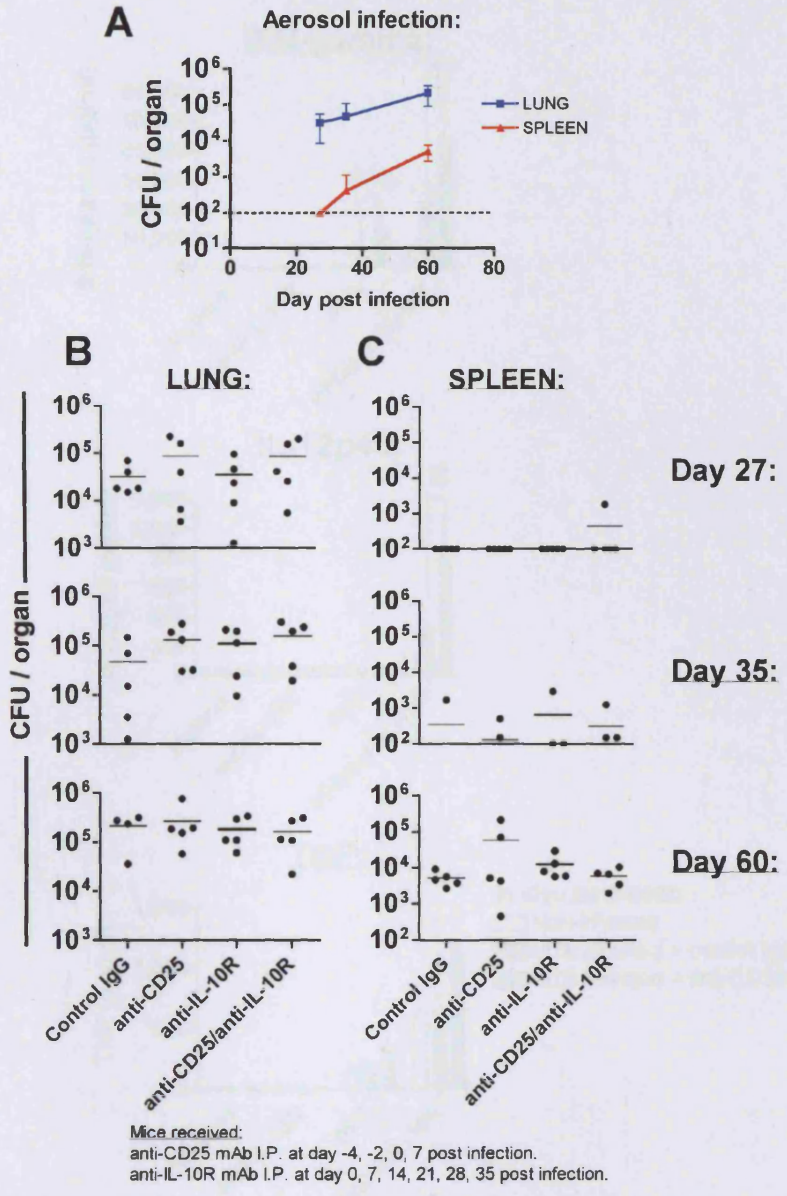


Figure 4.5: Depletion of CD25+ cells does not alter protection during early aerosol MTb infection.

(A) Female BALB/c mice were infected via the aerosol route with approximately 100 CFU H37Rv LSHTM strain and the bacterial burdens were monitored in the spleen and lungs throughout the time course. Mice were injected I.P. at days -4, -2, 0 and 7 post infection with GL113 (control IgG) or anti-CD25 (PC61; 0.5mg/mouse). Mice receiving anti-IL-10R mAb (1B1.3A; 0.5mg/mouse) were injected I.P. once weekly from the day of infection. At days 27, 35 and 60 post infection mice were killed by cervical dislocation and CFU were determined in the spleen **(B)** and lung **(C)**, as described in Materials and Methods (number of mice per group = 5). Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means.

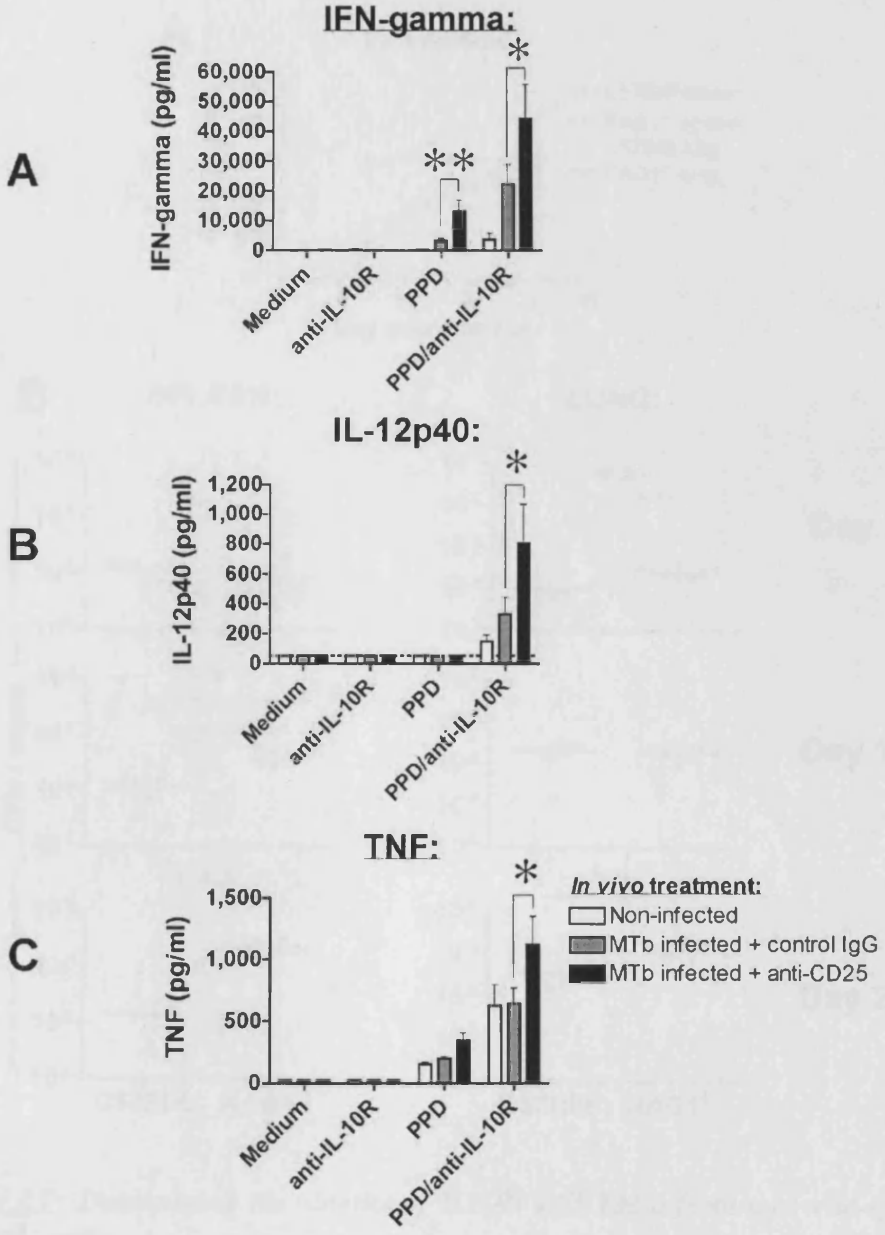


Figure 4.6: Despite no effect on bacterial load, depletion of CD25+ cells enhances pro-inflammatory cytokine responses to mycobacterial PPD.

Whole splenocytes were pooled from anti-CD25 treated mice or GL113 (control IgG) and re-stimulated *ex vivo* with PPD alone (10μg/ml) or in the presence or absence of anti-IL-10R mAb (both 10μg/ml). Supernatants were collected after 72 hours post stimulation and assayed by ELISA for **(A)** IFN-gamma, **(B)** IL-12p40, and **(C)** and TNF production. The effect of *in vivo* anti-CD25 mAb treatment was tested statistically using a One-Way ANOVA combined with a Bonferroni multiple comparison post-test (*, $P < 0.05$; **, $P < 0.01$).

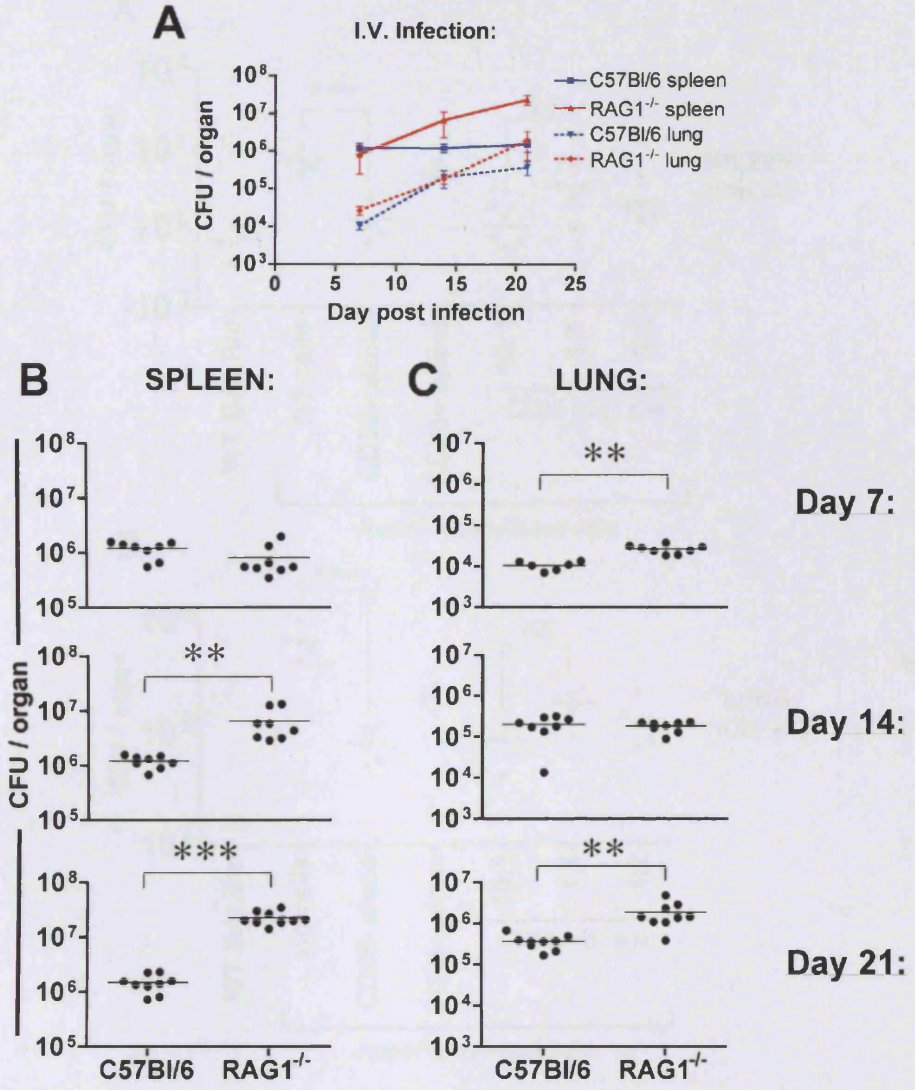


Figure 4.7: Determining the kinetics of H37Rv LSHTM infection in wild-type and B6.Rag1$^{-/-}$ mice.

(A) Wild-type B6 or B6.*Rag1*$^{-/-}$ mice were infected with 1x10^6 CFU via the I.V. route and bacterial burdens were monitored in the spleen and lungs throughout the time course. Mice were killed at the time-points indicated and bacterial load in the spleen **(B)** and lung **(C)**, were determined as described in Materials and Methods (number of mice per group = 8). Each point represents the CFU value from one mouse with the horizontal lines representing the geometric means. The difference in CFU between B6 and B6.*Rag1*$^{-/-}$ was tested statistically using a Students *t* test (**, P <0.01; ***, P <0.001).

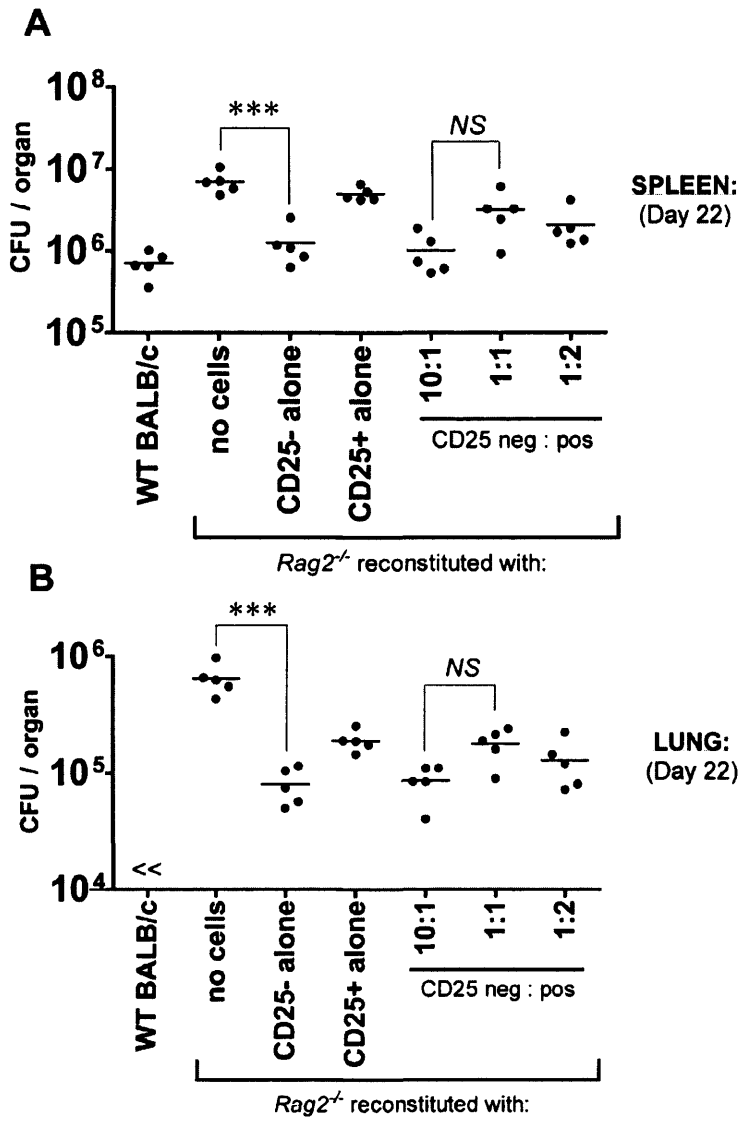


Figure 4.8: Increasing the natural ratio of naïve CD4+CD25- T cells to CD4+CD25+ Tregs plays a minimal role in regulating bacterial clearance.

CD25- and CD25+ T cells (All CD4+) were purified from the spleens of naïve BALB/c donors as described in materials and methods. BALB/c.*Rag2*$^{-/-}$ were reconstituted I.V. on day -1 before infection (cell number in brackets): *i)* PBS (no cells), *ii)* CD25- alone ($4x10^5$), *iii)* CD25+ alone ($4x10^5$), or combinations of CD25-: CD25+ *iv)* 10:1; ($4x10^5$:$4x10^4$), *v)* 1:1; ($4x10^5$:$4x10^5$), *vi)* 1:2; ($4x10^5$:$8x10^5$). Mice were infected I.V. with approximately $4x10^4$ CFU H37Rv LSHTM. At day 22 post infection mice were killed and CFU were determined in the spleen (A) and lung (B), as described in Materials and Methods (number of mice per group = 5). The effect of Treg transfer was tested statistically using a One-Way ANOVA combined with a Bonferroni multiple comparison post-test (*** P <0.001; *NS* = non-significant, << = below $1x10^4$ CFU). Results shown are representative of two independent experiments.

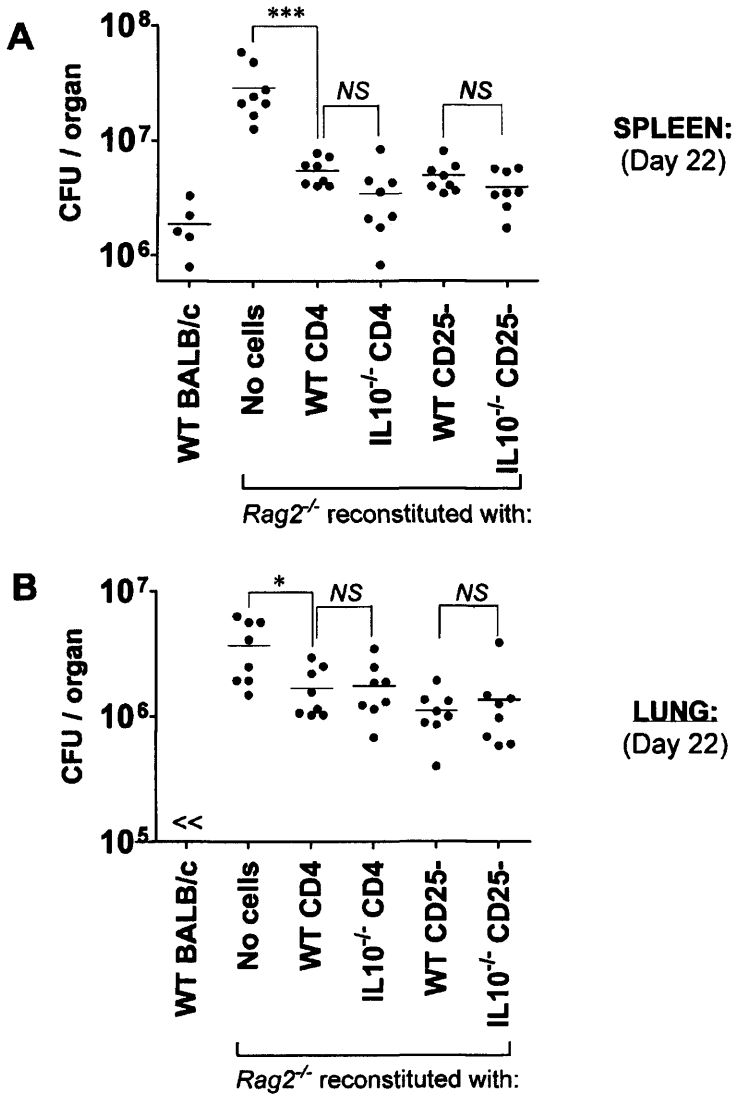


Figure 4.9: Whole CD4+ or CD4+CD25+ Treg derived IL-10 plays a minimal role in regulating the protective immune response to early MTb infection.

CD4+CD25- T cells were purified from the spleens of naïve WT BALB/c or BALB/c.*Il10*$^{-/-}$ mice as described in materials and methods. BALB/c.*Rag2*$^{-/-}$ mice were reconstituted I.V. the day before infection with $4x10^5$: i) no cells (PBS), ii) whole WT CD4+, iii) whole IL10$^{-/-}$ CD4+, iv) WT CD4+CD25-, v) IL10$^{-/-}$ CD4+CD25-. Mice were infected I.V. with approximately $1x10^5$ CFU H37Rv LSHTM. At day 22 post infection mice were killed and CFU were determined in the spleen **(A)** and lung **(B)**, as described in materials and methods (number of mice per group = 8). The effect of IL-10 was tested statistically using a One-Way ANOVA combined with a Bonferroni multiple comparison post-test (*, $P < 0.05$; ***, $P < 0.001$; NS = non-significant, << = below $1x10^4$ CFU). Results shown are representative of two independent experiments.

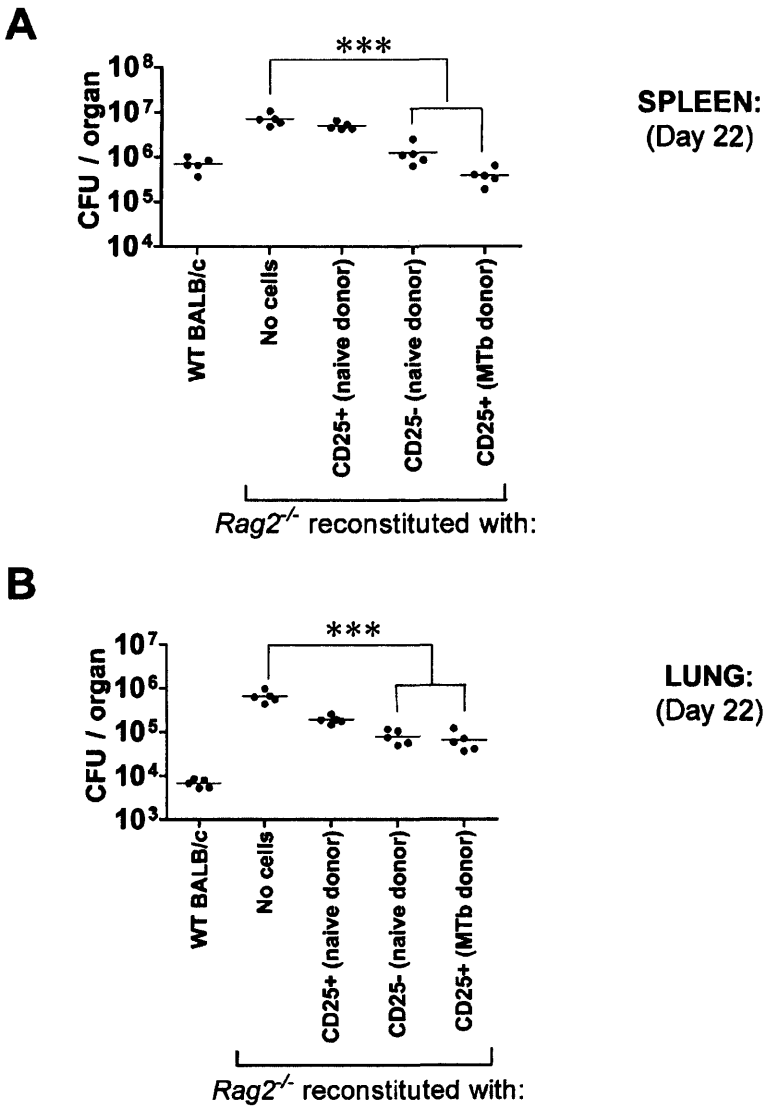


Figure 4.10: Antigen experienced CD4+CD25+ effector T cells mediate protection during early I.V. MTb infection.

To generate antigen experienced CD4+CD25+ T cells, BALB/c mice were infected I.V. with $1x10^5$ CFU H37Rv LSHTM strain. After 14 days, mice were killed by cervical dislocation, spleens removed and CD4+CD25+ T cells purified. For naïve donor cells, CD4+CD25+ and CD4+CD25- T cells were obtained from naïve BALB/c. For the transfer, BALB/c.*Rag2*$^{-/-}$ mice were reconstituted I.V. the day before infection with $4x10^5$: *i)* no cells (PBS), *ii)* naïve CD25-, *iii)* naïve CD25+, *iv)* antigen experienced CD25+. Mice were infected I.V. with approximately $4x10^4$ CFU H37Rv LSHTM strain. At day 22 post infection mice were killed and CFU were determined in the spleen (A) and lung (B), as described in Materials and Methods (number of mice per group = 5). The effect of T cell transfer was tested statistically using a One-Way ANOVA combined with a Bonferroni multiple comparison post-test (***, $P < 0.001$). Results shown are representative of two independent experiments.

4.7. Discussion:

Tregs mediate suppressive activity either via cell-cell contact or through production of suppressive cytokines such as IL-10 (Asseman *et al.*, 2003) or TGF-beta (Powrie *et al.*, 1996). The outcome limits aberrant autoimmune responses or may dampen over-exuberant and potentially pathological innate or adaptive immune responses to antigen or infectious challenge (Belkaid *et al.*, 2005). The presence of regulatory factors may benefit the host by limiting collateral damage but may inadvertently promote pathogen persistence as demonstrated in mouse models of *Toxoplasma* (Gazzinelli *et al.*, 1996) and *Trypanosomiasis* (Hunter *et al.*, 1997). Tregs have been subdivided into a) naturally occurring Tregs derived from the thymus or b) antigen driven IL-10 producing CD4+ Tregs (IL-10 Tregs) that result after exposure to specific stimulatory conditions during infection or antigen exposure (Mills, 2004, O'Garra *et al.*, 2004, Belkaid *et al.*, 2005). We sought to address the role of naturally occurring Tregs as regulators of the protective immune response to MTb by three different approaches. Firstly, by flow cytometry of the Tregs specific transcription factor FoxP3. Secondly, by antibody treatment of mice to deplete naturally occurring Tregs. Thirdly, using adoptive transfer of specific T cell populations into empty immuno-compromised hosts.

CD4+FoxP3+ Tregs increase in the lung following aerosol MTb infection:

To date a role for Tregs in regulating the immune response during murine MTb has not been defined. Our data demonstrates that Tregs play a limited role during early MTb infection. In agreement with the findings in human TB studies by Guyot-Revol *et al.*, (2006) and Ribeiro-Rodrigues *et al.*, (2006), we have identified an increase in

the percentage and absolute numbers of lung Tregs following aerosol exposure of mice to MTb. The numbers of lung CD4+FoxP3+ Tregs increased from day 8 post infection until day 29 post infection and was independent of the marker CD25 (Fig. 4.2C and D). The opposite kinetics were apparent for CD4+CD25+FoxP3- effector T cells (Fig. 4.2B), that started to increase during the latter stages of infection from day 29 to day 50 as reported by others (Junqueira-Kipnis *et al.*, 2004).

Treatment of mice with anti-CD25 mAb enhances pro-inflammatory cytokine production but does not correlate with enhanced bacterial clearance:

When using an antibody depletion model to address the involvement of Tregs in suppression of protective immunity to MTb thus promoting bacterial persistence and survival, we could assign no such role (Fig. 4.4). This finding is in accordance with the studies of Quinn *et al.*, (2006), who have also shown that anti-CD25 mAb treatment of mice following aerosol MTb (and BCG) infection had no effect on disease progression. In the same study treatment of mice with anti-CD25 during BCG infection enhanced IFN-gamma production. Similarly, in mice treated with anti-CD25 we observed an increase in IFN-gamma following *ex vivo* re-stimulation of cell suspensions with PPD.

As discussed earlier, depletion of CD4+CD25+ Tregs via anti-CD25 mAb therapy leaves a minor population of CD4+CD25-FoxP3+ Tregs that cannot be depleted. This subset of CD4+CD25-FoxP3+ Tregs will persist following anti-CD25 mAb treatment with the potential to regulate the immune response at the site of infection, thus masking any potential subtle effects on bacterial load. Whether or not CD4+CD25-FoxP3+ Tregs have a specific function in comparison to CD25+FoxP3+

Tregs during infection at this stage is not known. In addition, treatment of mice with anti-CD25 mAb may, depending on the half-life the antibody, distort the development later during infection of protective CD4+CD25+ effector T cells. We have demonstrated that during the first 14 days following I.V. infection CD4+CD25+ effector T cells are generated that mediate protection when adoptively transferred into naïve recipients and subsequently infected with MTb (Fig 4.10). In addition, we have recently acquired a FoxP3 knock-in green fluorescent protein (GFP) mouse thus enabling us to identify and track the development of FoxP3+ Treg populations including the cytokines they are producing during infection. Similarly this FoxP3-GFP mouse will enable us to obtain an ultra-pure population of CD4+FoxP3+ Tregs for our adoptive transfer studies, instead of enriching solely on the markers CD4 and CD25.

In contrast to anti-CD25 treatment alone, we were able to see an effect on bacterial load when mice were treated with a combination of both anti-CD25 mAb and anti-IL-10R mAb (Fig. 4.4B and C). This effect on bacterial load when both anti-CD25 and anti-IL10R were combined was enhanced compared to either mAb treatment alone (Chapter 3; Fig. 3.8). However, treatment of mice with both anti-CD25 mAb and anti-IL-10 mAb during aerosol MTb infection had no effect on bacterial load. The exact mechanism underlying the enhanced effect seen when combining treatment of anti-CD25 with anti-IL10R mAb during I.V. infection is at present not known. We originally postulated that in the absence of CD4+CD25+FoxP3+ Tregs IL-10 was still being produced by a minor subset of CD4+CD25-FoxP3+ Tregs. However, this hypothesis was disproved when transfer of CD4+ T cells from *Il10*$^{-/-}$ mice in to *Rag*$^{-/-}$ recipients, failed to influence bacterial burdens. IL-10 could be

from a non-T cell source such as macrophages and DC. As discussed in chapter 3, to address the possibility of the innate immune response as a source for IL-10 we are currently breeding B6.*Rag1*$^{-/-}$.*Il10*$^{-/-}$ mice.

TGF-beta as a potential immunosuppressive factor limiting the immune response to MTb:

Suppression of the immune response by TGF-beta is important in the down regulation of T cell mediated immune responses such as those that are needed to combat MTb infection. In mouse models of colitis TGF-beta has been shown to be an essential regulator of Treg activity and immune cells that express dominant negative TGF-beta receptors can escape regulatory control (Fahlen *et al.*, 2005). In mouse studies by Mogga *et al.*, (2003) it has been shown that TGF-beta expression was increased in the lungs of mice 12 weeks following aerosol MTb infection. When addressing the above roles for TGF-beta by antibody neutralisation, we found no such affect in a mouse model of early MTb infection. When anti-TGF-beta was administered in the presence of anti-CD25 mAb and anti-IL-10R mAb, no additive benefit on bacterial load was observed compared to mice that received combined anti-CD25 and anti-IL-10R mAb. The lack of effect observed by neutralisation of TGF-beta may reflect the complexity and context of its action, as TGF-beta can down-regulate Th1 and Th2 responses which may explain many of its suppressive effects (Li *et al.*, 2006b).

However, recent reports have demonstrated a pro-inflammatory role for TGF-beta plays in the derivation of pro-inflammatory Th-17 (IL-17 producing) T cells. It has been reported that stimulation of CD4+ T-cells in the presence of TGF-beta, IL-6

and TLR signalling drives the generation of Th-17 producing T cells (Veldhoen *et al.*, 2006, Bettelli *et al.*, 2006). To date, reports on the role of IL-17 in pro-inflammatory responses has drawn much attention in diseases including EAE (Park *et al.*, 2005; Langrish *et al.*, 2005) and MTb (Khader *et al.*, 2005). Furthermore, recent reports during BCG infection have shown that IL-17 production can be regulated by the presence of IFN-gamma (Cruz *et al.*, 2006). During murine MTb infection, gamma/delta T cells but not CD4+ T cells are the predominant producers of IL-17. In summary, the role of Th-17 in either protection or pathology in infectious disease is at present unclear.

Addressing the role of Tregs during MTb infection using an adoptive transfer model:

Treatment of mice with an antibody against the marker CD25 to deplete CD4+CD25+Tregs did not appear to influence bacterial burdens, this finding was also reported previously in MTb by Quinn *et al.*, (2006). With this in mind, we used an adoptive transfer model to address the role of Tregs in MTb infection as this model has been extensively used in murine colitis. We firstly established a suitable infection system in order to determine the kinetics of bacterial growth in the absence of the adaptive immune response. Following high does MTb infection, B6 mice controlled bacterial growth by day 7 showing no signs of disease. In contrast B6.*Rag1*$^{-/-}$ mice exhibited a dramatic increase in bacterial burdens that grew uncontrollably during the 21 days of infection. However, following adoptive transfer of CD4+CD25- T cells into B6.*Rag1*$^{-/-}$ mice, infection was controlled comparable to that observed in wild type mice. When CD4+CD25- T cells were transferred in the presence of increasing numbers of CD4+CD25+ Tregs (Fig. 4.8),

this had a limited effect on enhancing bacterial load that failed to reach statistical significance.

The negative effect seen on bacterial clearance was only observed when the Tregs were seeded at non-physiological ratios. In summary, our data implies that despite the observed increase in the number of lung Tregs following aerosol infection. Depletion of CD25+ cells and adoptive transfer studies failed to confirm a suppressive role for this regulatory subset at this early stage in MTb infection. Furthermore, during MTb infection Tregs may play a role in an antigen specific manner to inhibit the anti-mycobacterial response as demonstrated in murine studies of *Leishmania* (Belkaid *et al.*, 2002, Mendez *et al.*, 2004).

Chapter 5: The Role of Plasmacytoid pDC in Anti-Mycobacterial Immunity.

5.1. The role of plasmacytoid pDC in anti-mycobacterial immunity, aims of the investigation:

To address the role of plasmacytoid pDC in MTb we sought to:

1) Determine whether plasmacytoid pDC can become infected with MTb *in vitro* and induced to produce cytokines.

2) Identify the kinetics of plasmacytoid pDC influx to infected tissues following aerosol and I.V. MTb infection.

3) During the early stages of MTb infection, does the absence of plasmacytoid pDC alter the course of infection?

5.2. Background:

The importance of macrophages (Hart 1975; Schlesinger *et al.*, 1990; Gomes *et al.*, 1999; Underhill *et al.*, 1999, Gonzalez-Juerrero *et al.*, 2003) and more recently DC (Gonzalez-Juerrero *et al.*, 2001; Bodnar *et al.*, 2001; Tian *et al.*, 2005) in the immune response to MTb has been well documented. In contrast to macrophages, following phagocytosis of MTb DC are not able to mediate intracellular killing (Bodnar *et al.*, 2001). However, DC play a key role in uptake and antigen processing, migration to local draining lymph nodes and initiation of adaptive T-helper responses. The role of specific DC subsets in the immune response to MTb has to date not been documented.

Plasmacytoid pDC were originally described in human PBMC by Ronnblom *et al.*, (1983) as cells with a plasma cell like morphology. Recent studies in the mouse also identified a similar DC subset with identical morphology (Asselin-Paturel *et al.*,

2001; Nakano *et al.*, 2001; Bjork *et al.*, 2001). To date, numerous studies have shown that plasmacytoid pDC play a pivotal role in anti-viral immune response, as stimulation with Influenza A, murine CMV, or HSV resulted in the production of large quantities of type I IFN (Liu *et al.*, 2005). In the mouse, plasmacytoid pDC are considered immature DC because of their low expression of co-stimulatory molecules and the limited ability to stimulate naïve T-helper cells (Asselin-Paturel *et al.*, 2001). Because of this observation plasmacytoid pDC are regarded as classic innate cells. However, upon activation plasmacytoid pDC up-regulate co-stimulatory molecules and can present antigen. On the other hand plasmacytoid pDC have been shown to play a regulatory role under conditions such as allergy (de Heer *et al.*, 2004) or as mediators of vascularised graft tolerance (Ochando *et al.*, 2006). Furthermore, no studies at present have reported the ability of plasmacytoid pDC to either phagocytose bacteria or contribute during bacterial infection.

Interestingly, stimulation of mouse but not human plasmacytoid pDC with CpG-DNA induces the production of high levels of IL-12, IFN-alpha, but not IL-10 (Boonstra *et al.*, 2006). CpG-DNA is non-methylated (unlike human DNA) and is present in MTb as well as viruses and other organisms, therefore making it a potent inducer of pro-inflammatory cytokines such as IL-12. The involvement of plasmacytoid pDC may be important for inducing strong Th1 responses. Whilst Type I IFN's can co-operate in the Th1 response (Gautier *et al.*, 2005), they can sometimes be suppressive (Cousens *et al.*, 1997). Recent studies have shown enhanced immune responses to the intracellular pathogen *Listeria monocytogenes*, in the absence of Type I IFN (Auerbach *et al.*, 2004; Carrero *et al.*, 2004 and O'Connell *et al.*, 2004). The findings by Auerbach *et al.*, (2004) indicated that in the

absence of IFN-beta production, TNF production by phagocytic cells at the site of infection was increased thus promoting bacterial killing. Therefore the interplay between IL-12 and type I IFN's during infection may influence the disease outcome.

At present, no data is available on the ability of plasmacytoid pDC to be infected or activated by MTb or its products. In human studies plasmacytoid pDC have been shown by Cella *et al.*, (1999) to be present in the lymph nodes of TB patients and Lichtner *et al.*, (2005) have observed a decrease in the number of both classical DC and plasmacytoid pDC in the blood of active TB patients. Findings by Blasius *et al.*, (2004) demonstrated that following injection of mice in the footpad with heat-killed MTb, plasmacytoid pDC accumulated at the local draining knee lymph nodes. In addition, Manca *et al.*, (2001) have suggested that the hyper-virulent phenotype of the clinical isolate MTb HN878 in mice is due to induction of type I IFN. However, the source of Type I IFN was not examined.

The involvement of plasmacytoid pDC in immunity to pathogens can be direct as shown by its potent anti-viral effects mediated by Type I IFN, or indirectly via IFN-alpha/beta inhibition of IL-12, as discussed above. Similarly as discussed above, Plasmacytoid pDC may also play a regulatory role under certain conditions e.g. in allergy (de Heer *et al.*, 2004), although at present through unknown mechanisms. In this study, we examined whether plasmacytoid pDC play a role during MTb infection *in vivo* as well as *in vitro*.

5.3. Does MTb infect plasmacytoid pDC?

5.3.1. In vitro infection of BM macrophages and BM myeloid DC but not plasmacytoid pDC induces pro-inflammatory cytokine production:

To determine whether plasmacytoid pDC could be infected with MTb *in vitro* and induced to produce cytokines, plasmacytoid pDC were infected alongside BM macrophages and myeloid DC as these cell types have already been shown to be infected with MTb and subsequently produce pro-inflammatory cytokines. Before we could address this, we firstly established an *in vitro* infection system whereby the infection dose of MTb on BM myeloid DC was titrated at MOI of 1:1, 5:1 and 10:1 (MTb:DC). The titration of MTb indicated that the optimal MOI for infection of DC was 5:1 as it gave significant infection, strong cytokine induction but with low DC death. This finding was in contrast to infections using an MOI of 1:1 where only low levels of cytokine were induced, or an MOI of 10:1, where 90% of DC where non-viable within 24 hours. Macrophages (Fig. 5.1A), myeloid DC (Fig. 5.1B) and Flt-3L cultured plasmacytoid pDC (Fig. 5.1C) were derived from the BM or alternatively organ derived plasmacytoid pDC were freshly isolated from the spleen (Fig. 5.1D), as described in Materials and Methods.

Following cultures of BM and splenic derived plasmacytoid pDC for 24 hours in the presence of MTb, no detectable levels of IL-12p70, IL-12p40, TNF and IFN-alpha were produced (Fig. 5.2A). However, in response to CpG-B DNA stimulation, both splenic and BM derived plasmacytoid pDC produced substantial levels of IL-12p70, IL-12p40, TNF and IFN-alpha, but no IL-10 (Fig. 5.2B), as reported by others (Asselin-Paturel *et al.*, 2001; Boonstra *et al.*, 2006). In contrast to plasmacytoid

pDC, culture of BM macrophages and BM myeloid DC in the presence of MTb induced the pro-inflammatory cytokines IL-12p40 and TNF after 24 hours (Fig. 5.2A) as reported by others (Bodnar *et al.*, 2001). CpG-B DNA stimulation of BM macrophages and BM myeloid DC produced high levels of IL-12p40, low levels of TNF and no detectable IL-12p70 or IFN-alpha (Fig. 5.2B).

To determine if plasmacytoid pDC were able to phagocytose and consequently be infected by MTb, the growth of bacilli was determined after 24 hours of culture following infection at an MOI of 5:1 (MTb:APC). When intracellular bacterial counts were determined, progressive growth within BM macrophages (Fig.5.3A) and BM myeloid DC (Fig.5.3B) was observed as reported by others (Bodnar *et al.*, 2001). In contrast, plasmacytoid pDC were only minimally infected with MTb (Fig. 5.3C).

5.4. Tracking the kinetics of plasmacytoid pDC *in vivo* following MTb infection:

5.4.1. Plasmacytoid pDC are detected in spleen and lung suspensions by the mAb 120G8:

Our data from *in vitro* studies thus far suggests that plasmacytoid pDC are not infected with MTb *in vitro* and produce no detectable levels of cytokine. We therefore examined the kinetics of plasmacytoid pDC following *in vivo* infection of mice using flow cytometry by tracking expression of the plasmacytoid pDC specific marker 120G8. We wanted to determine if plasmacytoid pDC were attracted to the site of infection and inflammation as previously suggested for heat-killed MTb

(Blasius *et al.*, 2004) and in human MTb studies (Cella *et al.*, 1999). Asselin-Paturel *et al.*, (2003) described the 120G8 mAb that recognises an unknown epitope that is highly expressed on the surface of murine plasmacytoid pDC. In addition, 120G8 is also expressed on activated B cells and some CD11c+ populations albeit at low expression levels.

Prior to our studies, we firstly conjugated the 120G8 mAb to the fluorochrome Alexa-488, before it was tested *in vitro* and used *in vivo* for flow cytometry studies. To identify plasmacytoid pDC, whole splenocytes from 129/Sv mice were stained for expression of B220+, CD11c+, GR1+ (Fig. 5.4A), or with CD11c and 120G8 (Fig. 5.4B). The percentages of splenic plasmacytoid pDC using these two different staining procedures were identical (Fig. 5.3B). Similar to the spleen, a defined population of plasmacytoid pDC (CD11c+120G8+) were identified in the lung (Fig. 5.4C). Upon closer examination of the lung CD11c high population (CD11c+120G8-), we observed that these constituted a heterogeneous population consisting potentially of both large alveolar macrophages and smaller lung DC as determined by the forward scatter (FSC) and side scatter profiles (SSC) (Fig. 5.4C). This observation is in keeping with the detailed phenotypic analysis of lung CD11c+ populations performed by de Heer *et al.*, (2004).

Interestingly, the percentage of plasmacytoid pDC varies between mouse species with 129/Sv mice having the highest percentage of plasmacytoid pDC when compared to BALB/c and B6 as reported by others (Asselin-Paturel *et al.*, 2003). We therefore confirmed this finding by analyzing the percentage of splenic plasmacytoid pDC in the 129/Sv, BALB/c and C57Bl/6 (Fig 5.5A). In addition to

the 129/Sv mouse having the highest percentage of plasmacytoid pDC, this finding was also reflected in the serum levels of IFN-alpha induced following I.V. challenge with CpG-B DNA (Fig 5.5B).

5.4.2. The number of plasmacytoid pDC do not increase in the spleen or lung following early I.V. MTb infection:

We identified whether plasmacytoid pDC accumulated at the site(s) of infection following MTb infection. To address this, mice were infected I.V. with 10,000 CFU H37Rv and the lungs (Fig. 5.6A) and spleen (Fig. 5.6B) were prepared and analysed on day 0, 1, 12, 21 and 43 after infection by flow cytometry for the expression of 120G8 and CD11c and compared to non-infected control mice. Dyuring the course of infection (Fig. 5.7A), the absolute numbers of plasmacytoid pDC in both the lungs (Fig. 5.7B) and spleen (Fig. 5.7C), remained unchanged during the course of I.V. infection as compared to non-infected control animals. However, during the latter stages of infection (day 43), a minor increase in the numbers of total plasmacytoid pDC (CD11c+120G8+) in both the lungs (Fig. 5.7B) and spleen (Fig. 5.7C) became apparent. Our findings suggest that following I.V. infection; the small increase in plasmacytoid pDC at day 43 suggests that they may play a role during the latter stages of MTb infection.

5.4.3. Plasmacytoid pDC do not accumulate in the lung following aerosol infection:

MTb infection of mice by the I.V. route did not result in an increase of plasmacytoid pDC in the lung or spleen during the early stages of infection. This finding may be a

reflection on *i)* the I.V. route of MTb infection in mice (that reflects a model of disseminated TB rather than pulmonary TB) and *ii)* the large numbers of MTb injected I.V. Both these factors may mask any subtle responses or role for plasmacytoid pDC during infection. We therefore infected mice via the aerosol route with a low dose of MTb in order to establish a model of pulmonary TB.

BALB/c mice were infected via the aerosol route with approximately 100 CFU MTb, and at specific time-points post infection bacterial burdens were determined (Fig. 5.8A). The percentages (Fig. 5.8) and absolute numbers of lung CD11c high cells (Fig 5.9A) and plasmacytoid pDC (Fig. 5.9B) did not change on day 2, 8, 14 and 29 post infection. In contrast to the lung APC populations, the number of lung CD4+ T cells increased from day 8 post infection (Fig. 5.9C) and remained elevated throughout the infection time course. In the spleen, we observed no increase in splenic plasmacytoid pDC (CD11c+120G8+), splenic DC (CD11c+120G8-) or CD4+ T cells during the course of infection (data not shown). This observation may reflect the slow dissemination of MTb from the site of infection to secondary organs following infection via the aerosol route.

Our data suggests that plasmacytoid pDC do not accumulate in the lung following early aerosol infection, but the accumulation of plasmacytoid pDC may occur during the latter stages of infection as seen from day 43 post I.V. infection (Fig. 5.7). In addition, our approach focused specifically on DC influx into the lungs or spleen, it did not however take into account the potential influx of this DC subset to the local draining lymph nodes.

5.5. The immune response to MTb infection is unaffected in the absence of plasmacytoid pDC:

5.5.1. The antibody 120G8 depletes steady state splenic plasmacytoid pDC:

Depletion studies in the mouse by de Heer *et al.*, (2004) have shown a regulatory role for lung plasmacytoid pDC in limiting asthmatic responses to harmless inhaled antigen. Therefore to address a role for plasmacytoid pDC in regulating the immune response at the site of MTb infection, we treated 129/Sv mice *in vivo* with 120G8 mAb. As others and we have shown (Asselin-Paturel *et al.*, 2003), 129/Sv mice possess a higher percentage of plasmacytoid pDC than other mouse strains including BALB/c and B6 mice (Fig. 5.5A). This observation was reflected by the level of serum Type I IFN induced following I.V. challenge with CpG-B DNA (Fig. 5.5B). With this difference in mind, we postulated that depletion of plasmacytoid pDC in 129/Sv mice, as opposed to BALB/c or B6 mice, would potentially have a greater influence on bacterial burdens.

To test the efficacy of plasmacytoid pDC depletion *in vivo*, naïve 129/Sv mice received two injections I.P. at 24 hour intervals of either 120G8 mAb (1mg/mouse) or control IgG (GL113). Six hours following the second injection the mice were killed and spleens were analysed by flow cytometry for the percentage of plasmacytoid pDC. Flow cytometric analysis of B220+CD11c+GR1+ plasmacytoid pDC in control treated mice (Fig. 5.10A) and 120G8 mAb treated mice (Fig. 5.10B), revealed an >80% depletion in splenic plasmacytoid pDC.

5.5.2. Depletion of plasmacytoid pDC in vivo has no effect on regulating bacterial clearance during early MTb infection:

Next, we examined if plasmacytoid pDC play a regulatory role during MTb infection in mice by *in vivo* antibody depletion during the early immune response to MTb. Prior to infection 129/Sv mice were treated I.P. with either 120G8 mAb (1mg/mouse) or control IgG (GL113) at day -1 and day 0. Mice were then infected I.V. with either 50,000 CFU (Fig 5.11A) or 250,000 CFU (Fig 5.11B) MTb. Mice continued receiving once weekly I.P. injections of 120G8 mAb until the end of the infection time course. At day 18 post infection, mice were killed and bacterial burdens in the spleen (Fig. 5.11C) and lungs (Fig. 5.11D) were determined. Following 120G8 depletion of plasmacytoid pDC there was no apparent effect on the course of infection in either the spleen or lung compared to control IgG treated mice irrespective of the initial infection dose.

The lack of effect seen on bacterial loads may reflect the route of infection. As discussed above, I.V. injection with large non-physiological MTb doses may mask any subtle effects that plasmacytoid pDC may have on regulating bacterial clearance. Therefore to further address the role of plasmacytoid pDC during early infection, similar experiments need to be repeated in mice infected with a low dose of MTb via the more physiological aerosol route to reflect pulmonary TB.

5.6. Figures:

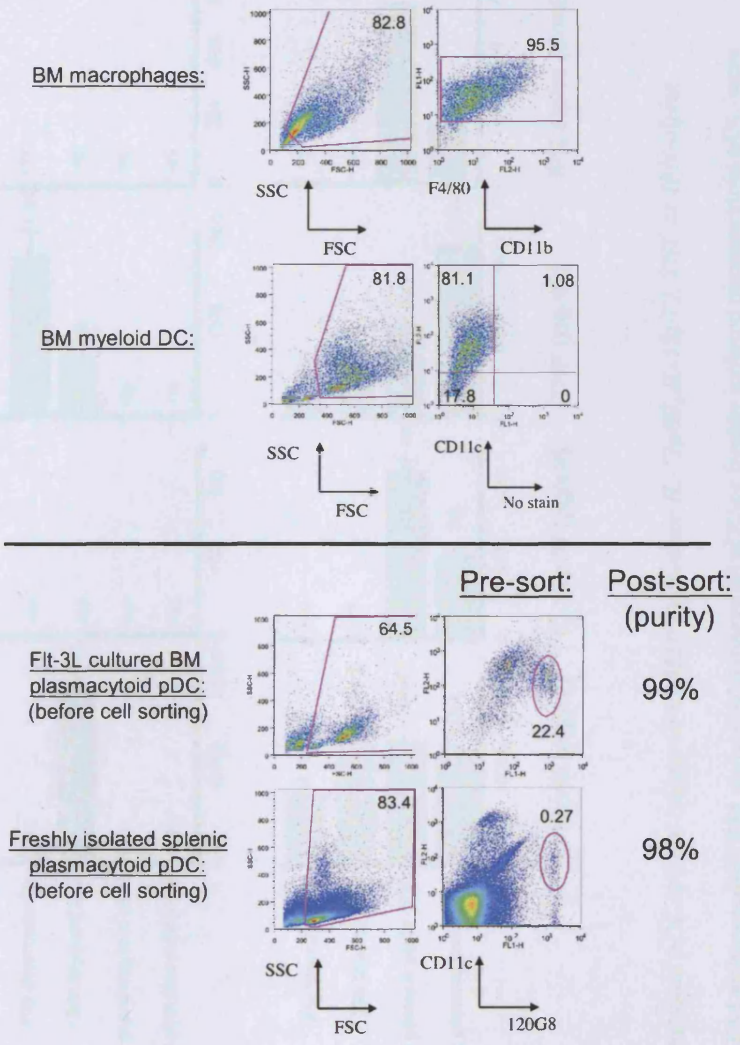


Figure 5.1: Phenotypic analysis of macrophages and DC cultured either from the BM or freshly isolated from the spleen.

(A) BM macrophages (CD11b+F4/80+), **(B)** BM myeloid DC (CD11c+), and **(C)** plasmacytoid pDC (CD11c+120G8+) were either cultured from the BM or alternatively **(D)** plasmacytoid pDC (CD11c+120G8+) were freshly isolated form the spleen. The cells were stained with the appropriate flow cytometry antibodies as described in Materials and Methods. The BM macrophages and BM myeloid DC were not FACS sorted and used in infections straight from cell culture due to high cell purity. However, the BM plasmacytoid pDC were FACS sorted after culture in order to obtain an ultra-pure population of homogenous DC. Similarly, splenic plasmacytoid pDC were freshly isolated from the spleen by FACS sorting.

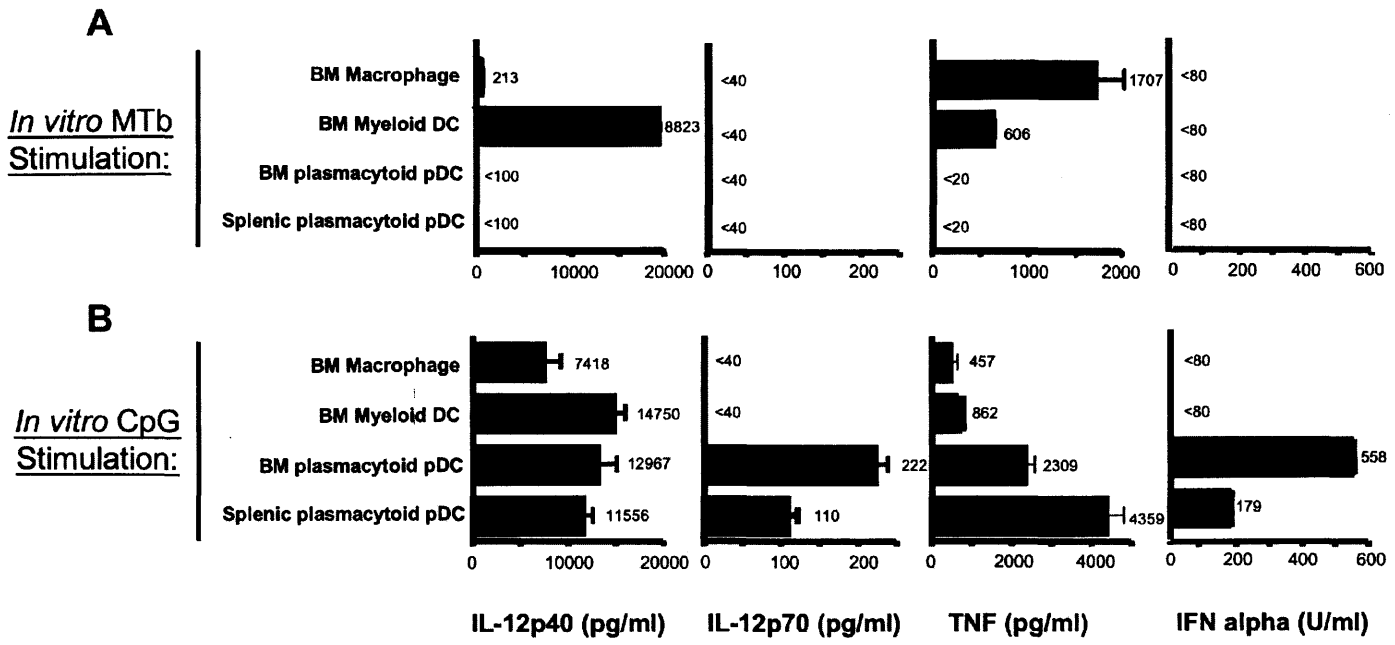


Figure 5.2: Plasmacytoid pDC are not triggered by MTb to produce IL-12p40, IL-12p70, TNF or IFN-alpha.

BM myeloid DC, BM macrophages, BM derived plasmacytoid pDC or freshly isolated plasmacytoid pDC were obtained as described in Materials and Methods. Cells were stimulated *in vitro* with either **(A)** live MTb H37Rv at a multiplicity of infection of 5:1 (MTb:DC) or **(B)** CpG-B DNA (1668 ISS; 1μM) for 24 hours. After 24 hours supernatants were harvested and analyzed by immunoassay. Results shown are representative of 3 independent experiments.

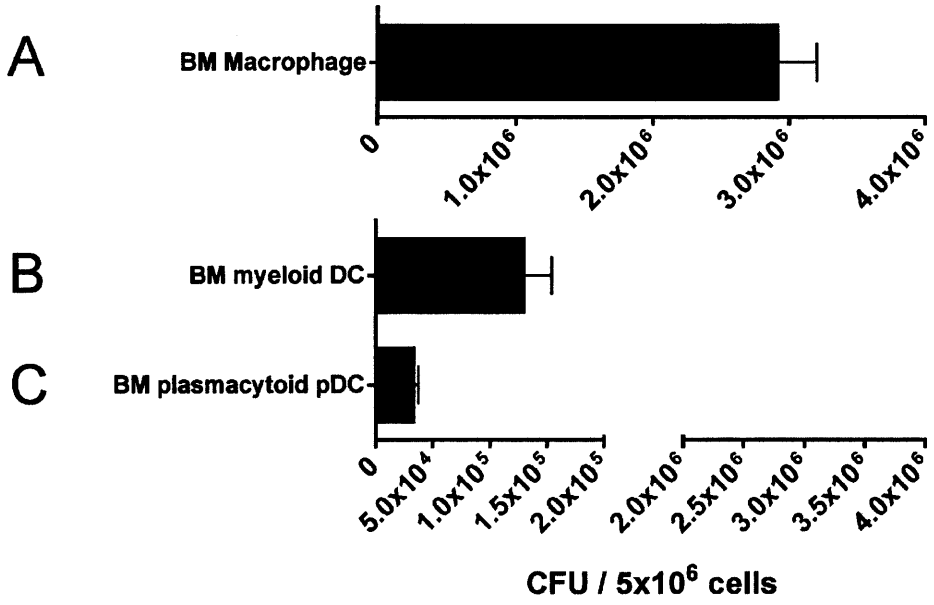


Figure 5.3: BM macrophages, BM myeloid DC but not BM plasmacytoid pDC are infected with MTb in vitro.

(A) BM macrophages or (B) BM myeloid DC and (C) BM plasmacytoid pDC were derived from BALB/c mice as described in Materials and Methods. Following culture, APC ($5x10^6$ APC) were infected with H37Rv at an MOI of 5:1 (MTb:APC). After 24 hours cells were harvested and CFU performed in as described in Materials and Methods.

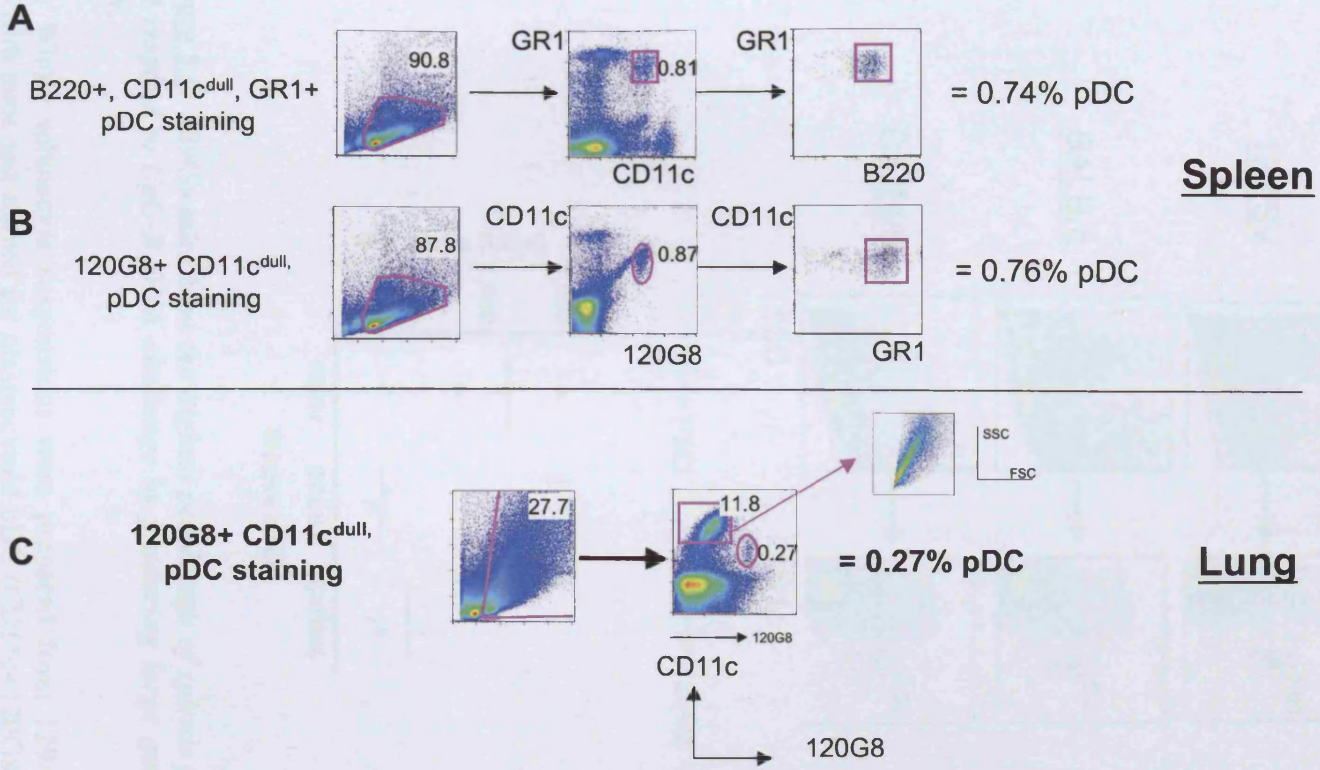


Figure 5.4: Plasmacytoid pDC are dectected in both spleen and lung suspensions by 120G8 mAb.

Whole splenocytes or lung cells from naïve 129/Sv mice were prepared and stained as described in Materials and Methods. Expression of plasmacytoid pDC in the spleen was shown by analysis of **(A)** B220, CD11c and GR1 or **(B)**120G8, CD11c and GR1. Plasmacytoid pDC were also analysed in the lung by expression of **(C)**120G8 and CD11c. All percentages shown are of total live cells and are representative of 3 individual mice.

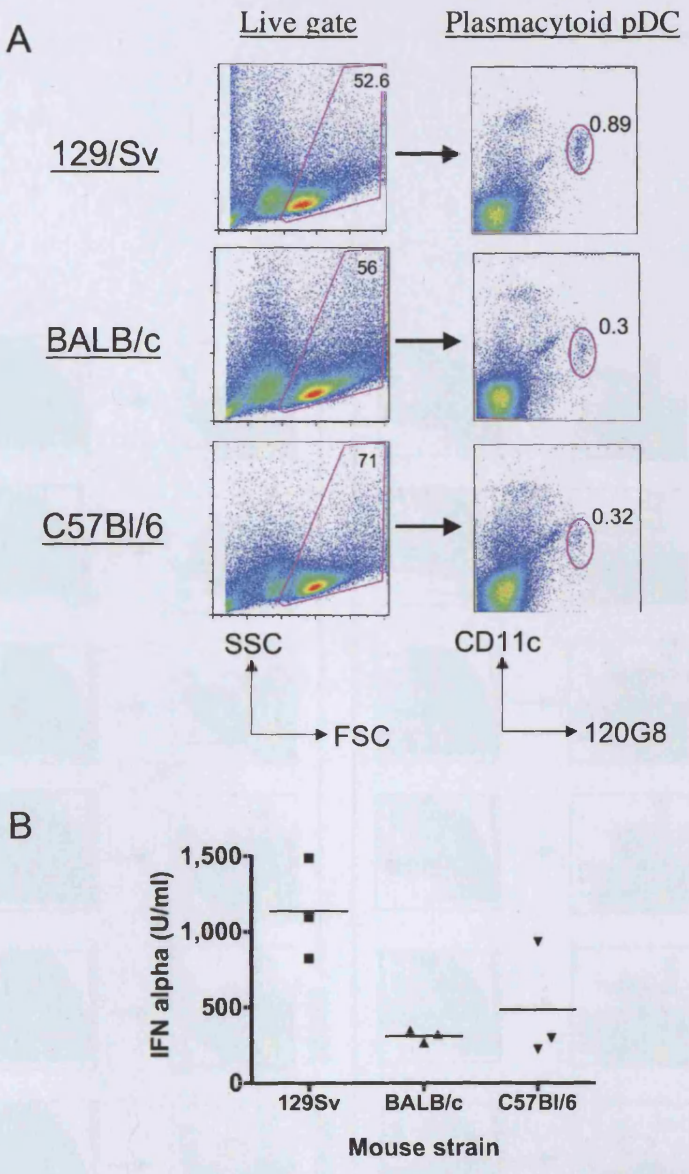


***Figure 5.5**: 129/Sv mice have the highest percentage of splenic plasmacytoid pDC and respond to CpG-B DNA challenge by producing large quantities of Type I IFN.*

(A) Whole splenocyte suspensions were prepared from 129/Sv, BALB/c and C57Bl/6 mice and stained for plasmacytoid pDC (CD11c+120G8+) as described in Materials and Methods. **(B)** Mice were challenged I.V. with 5μM CpG-B DNA (1018 ISS; 5μM), for a period of 6 hours before serum was taken and analysed by ELISA for IFN-alpha. Each point represents the serum level of IFN-alpha from one mouse with the horizontal lines representing the geometric means.

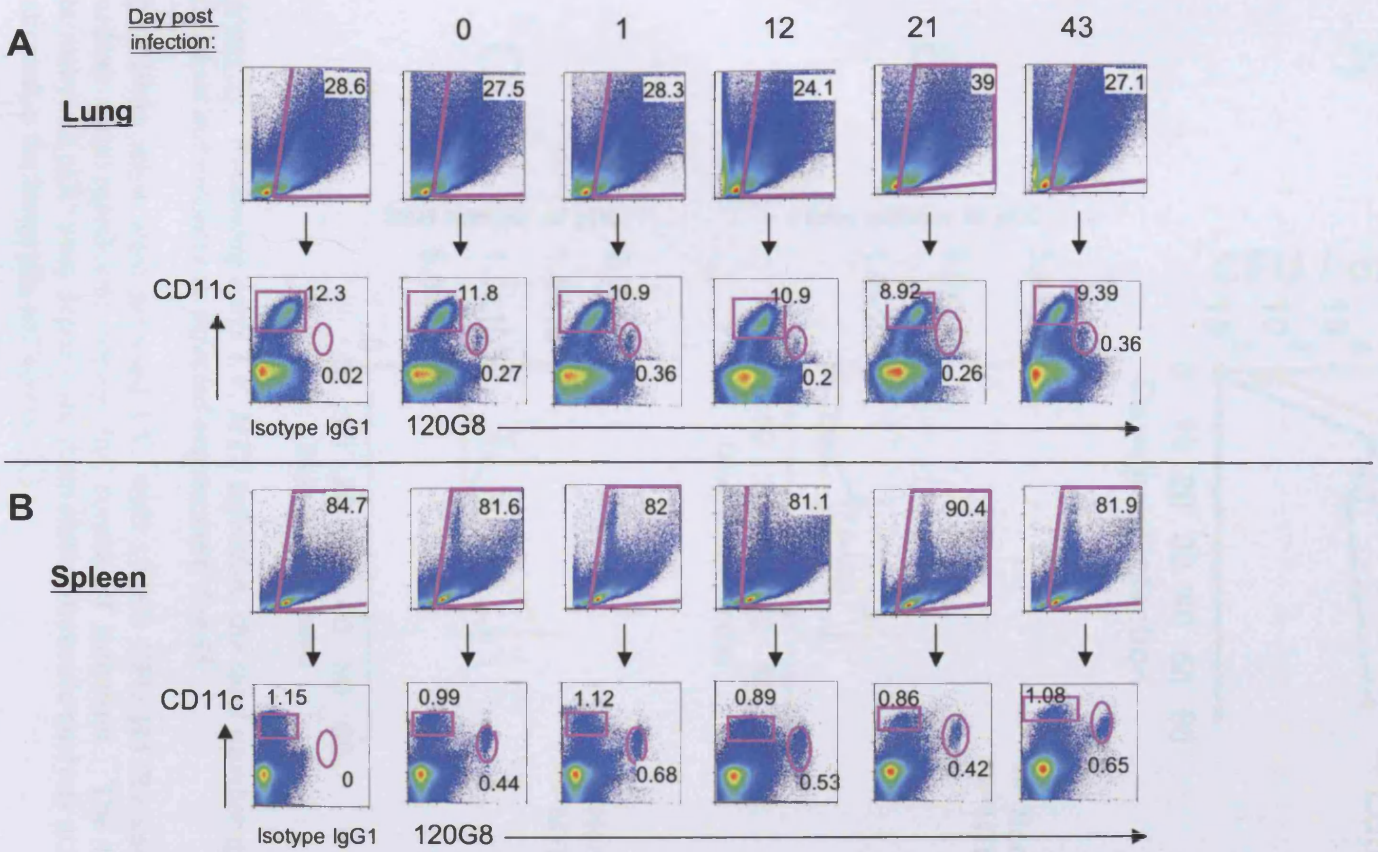


Figure 5.6: Following early I.V. MTb infection, plasmacytoid pDC do not accumulate in infected organs.

129/Sv mice were infected I.V. with 10,000 CFU H37Rv. At the time points indicated the level of CD11c and 120G8 expression in the **(A)** lungs and **(B)** spleen were analysed by flow cytometry). All percentages shown are of total live cells and are representative of 3 individual mice.

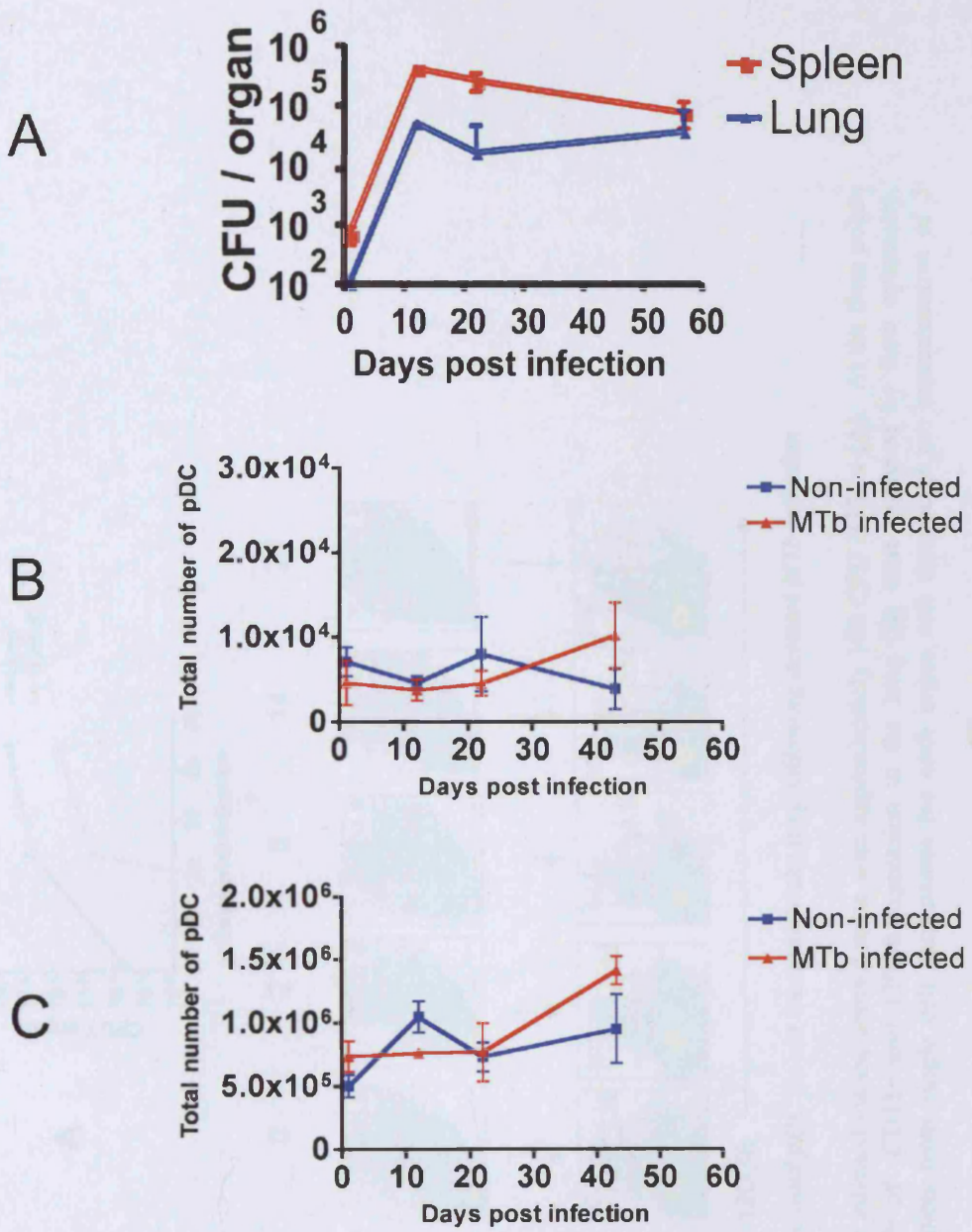


Figure 5.7: Following early I.V. MTb infection, the total number of plasmacytoid pDC does not increase in infected organs until day 43.

(A) 129/Sv mice were infected I.V. with 10,000 CFU H37Rv and the bacterial burdens were monitored during the course of infection. The total levels of plasmacytoid pDC were determined from flow cytometric analysis at the time-points indicated in the lungs (B) and spleen (C).

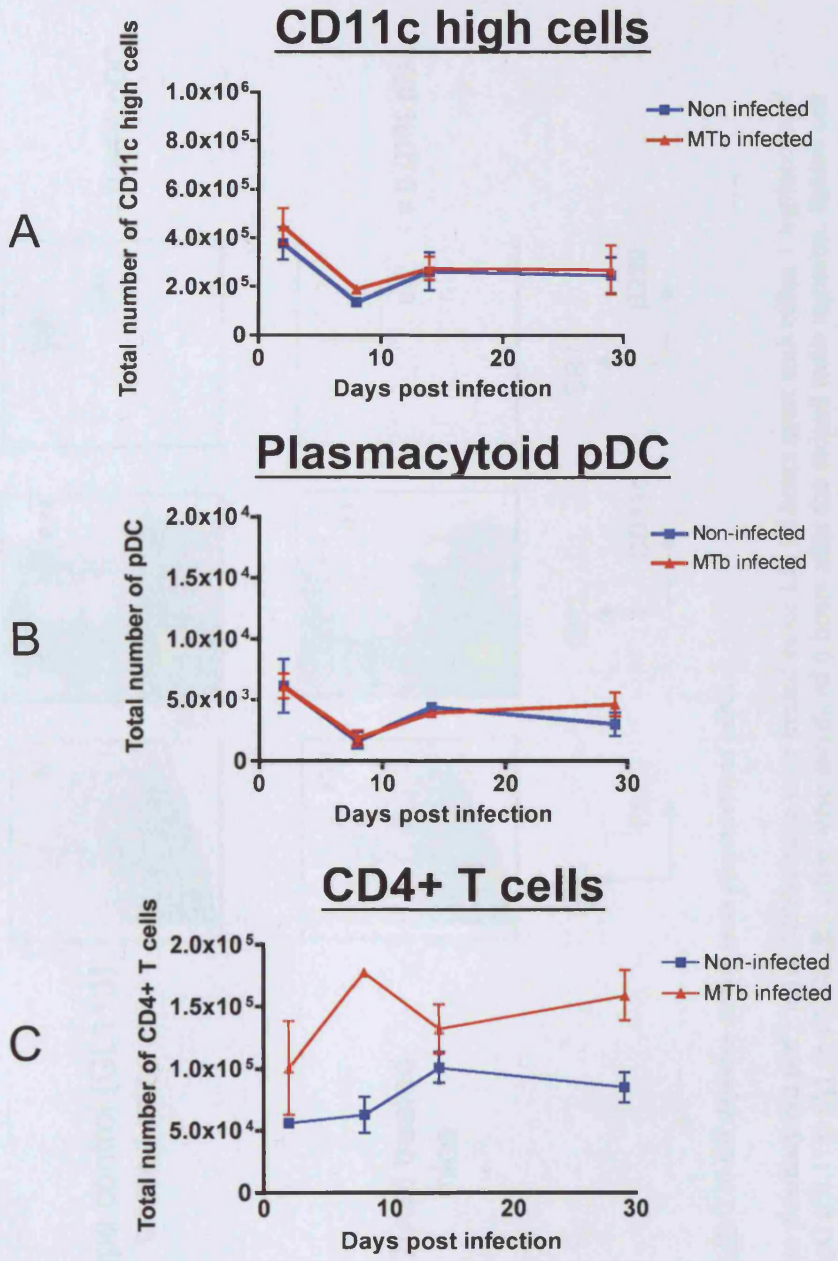


***Figure 5.9*: The absolute numbers of plasmacytoid pDC do not increase in the lung following aerosol MTb infection.**

BALB/c mice were infected via the aerosol route with approximately 100 CFU H37Rv. At the time points indicated the total numbers of **(A)** classical DC (CD11c+120G8-), **(B)** plasmacytoid pDC (CD11c+120G8+) and **(C)** CD4+ T cells were determined from flow cytometric data.

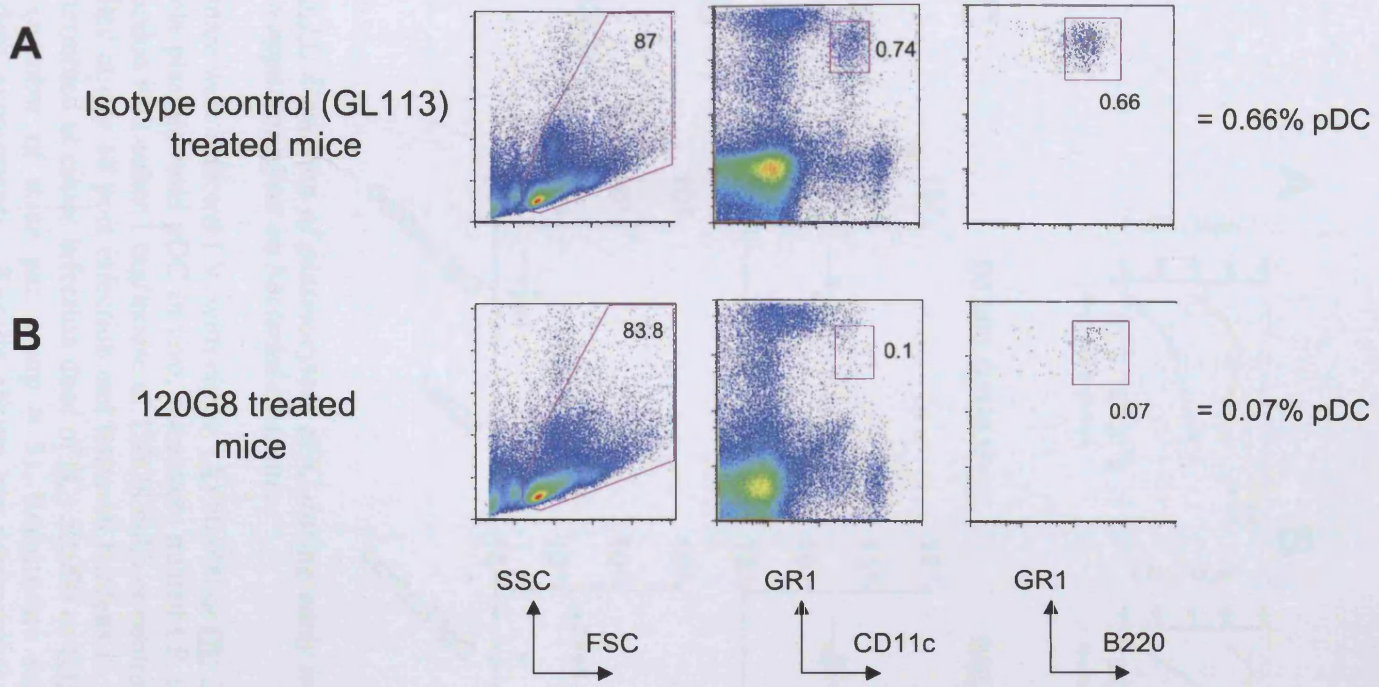


Figure 5.10: 120.G8 depletes steady state plasmacytoid pDC.

To deplete plasmacytoid pDC; naïve 129/Sv mice were treated twice I.P. 24 hours apart with either 1 mg/mouse of control IgG (GL113) **(A)** or 120G8 **(B)**. Mice were sacrificed 6 hours after the second mAb injection. Spleen cell suspensions were analyzed for plasmacytoid pDC levels by flow cytometry. All percentages shown are of total live cells and are representative of 3 individual mice. Results shown are representative of 3 independent experiments.

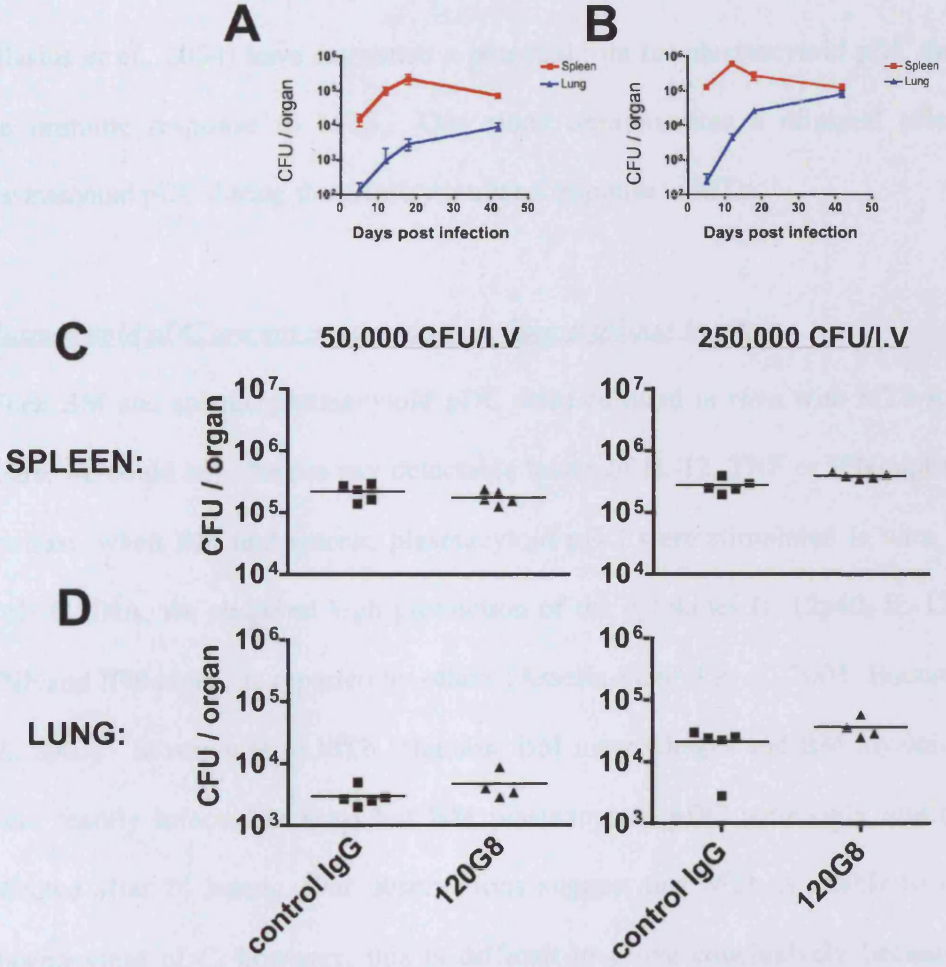


***Figure 5.11*: Depletion of plasmacytoid pDC during early in vivo MTb infection shows no apparent effect on bacterial clearance.**

129/Sv mice were infected I.V. with either: **(A)** 50,000 or **(B)** 250,000 CFU H37Rv. To deplete plasmacytoid pDC *in vivo*, mice were treated I.P. at day -1, 0, 7 and 14 post infection with either 1 mg/mouse of 120G8 mAb or control IgG (GL113). Mice were killed at day 18 post infection and bacterial burdens in the spleen and lungs were determined at either infection dose of **(C)** 50,000 or **(D)** 250,000 CFU MTb H37Rv (number of mice per group = 5). Results are representative of two independent experiments. Results shown are representative of 2 independent experiments.

5.7. Discussion:

Recent studies in the human (Cella *et al.*, 1999; Lichtner *et al.*, 2005) and mouse (Blasius *et al.*, 2004) have suggested a potential role for plasmacytoid pDC during the immune response to MTb. Our study demonstrates a minimal role for plasmacytoid pDC during the primary immune response to MTb.

Plasmacytoid pDC are not triggered to produce cytokines in vitro:

When BM and splenic plasmacytoid pDC were cultured *in vitro* with MTb for 24 hours, we could not observe any detectable levels of IL-12, TNF or IFN-alpha. In contrast, when BM and splenic plasmacytoid pDC were stimulated *in vitro* with CpG-B DNA, we observed high production of the cytokines IL-12p40, IL-12p70, TNF and IFN-alpha, as reported by others (Asselin-Paturel *et al.*, 2001; Boonstra *et al.*, 2003). In response to MTb infection, BM macrophages and BM myeloid DC were readily infected *in vitro* but BM plasmacytoid pDC were only minimally infected after 24 hours. Our observations suggest that MTb is unable to infect plasmacytoid pDC, however, this is difficult to prove conclusively because the viability of plasmacytoid pDC decreases profoundly from around 12 hours in culture, which was independent of the stimulus. It has been reported by Asselin-Paturel *et al.*, (2001) that plasmacytoid pDC undergo apoptosis in the absence of a strong activation signal, such as that afforded by CpG-DNA. It is therefore possible that MTb (or its CpG-DNA) may sub-optimally activate plasmacytoid pDC, but this activation is not strong enough to induce cytokine production or inhibit the apoptosis pathway. Furthermore, due to the difficulties encountered with the viability of plasmacytoid pDC in culture, Ziehl Nielsen staining of plasmacytoid pDC from

infection cultures did not allow us to determine whether this DC subset could be infected *in vitro*. With this observation in mind and the findings by Cella *et al.*, (1999) that plasmacytoid pDC were present in human tonsil tissue, we addressed the role of plasmacytoid pDC during *in vivo* MTb infection by antibody depletion

The kinetics of plasmacytoid pDC following MTb infection:

During aerosol MTb infection, the numbers of plasmacytoid pDC and CD11c+120G8- populations in the lungs and spleen did not significantly increase. However, levels of lung CD4+ T cells increased after day 8 following aerosol infection as previously published by others (Feng *et al.*, 1999, Serbina and Flynn, 1999, Lazarevic *et al.*, 2005). During I.V. MTb infection the levels of plasmacytoid pDC did not appear to increase until day 43 post infection. In our studies, we did try to address the potential role for plasmacytoid pDC migrating to the local lung draining lymph nodes, but due to the constraints of working under bio-safety level 3 conditions, this made accurately dissecting the lung draining lymph nodes very difficult and was therefore not assessed.

Our findings are in contrast with studies by Blasius *et al.*, (2004), when injection of heat-killed MTb into the footpad subsequently gave rise to the accumulation of plasmacytoid pDC in the local draining lymph nodes. The differences observed between the studies of Blasius *et al.*, (2004) and our own may be linked to key differences in immunogenicity between heat-killed and live virulent MTb, the site of infection (e.g. lung or lymph node), and the route of MTb administration. Another hypothesis for the accumulation of plasmacytoid pDC observed by Blasius *et al.*, (2004) may be the presence of CpG-DNA or other contaminating immunogenic

pathogen related products present in the heat-killed MTb preparation which were not readily accessible in the live organism.

There are a number of reasons that in our studies APC did not increase in the lung following aerosol MTb infection, these factors include the site of infection (e.g. draining lymph node) and the time following infection. Following aerosol MTb infection Gonzalez-Juarrero *et al.*, (2003), have reported a minor increase in lung DC and macrophage populations in the first 20 days post infection. In addition, studies by Humphreys *et al.*, (2006) by infecting mice intra-nasally with a GFP-tagged BCG have shown that lung DC play a central role in the dissemination of bacteria from the site of infection to the draining mediastinal lymph nodes. In our study, lung DC may have been constantly cycling between the lung and draining lymph nodes potentially masking any observed increase or decrease on total cell numbers.

Depletion studies did not address a role for plasmacytoid pDC in vivo:

As we were unable to infect or induce cytokines following infection *in vitro*, or observe any increase in the total number of plasmacytoid pDC following MTb infection *in vivo,* we further evaluated the role of plasmacytoid pDC during MTb infection by antibody depletion *in vivo*. In line with this, studies in the mouse by de Heer *et al.*, (2004) have shown a regulatory role for lung plasmacytoid pDC in limiting asthmatic responses to harmless inhaled antigen. In contrast, in this study depletion of plasmacytoid pDC before MTb infection, revealed no regulatory role for this DC subset during the primary immune response to MTb in the lung. We did not address whether plasmacytoid pDC play a role during chronic infection when death of bacteria may be greater, i.e. during memory responses or following antibiotic

treatment. Indeed, it has been shown that MTb contains CpG-DNA that can activate DC *in vitro* (Bafica *et al.*, (2005), and during bacterial killing the presence of CpG-DNA could activate plasmacytoid pDC via interaction with endosomally expressed TLR-9. Therefore, the role of plasmacytoid pDC needs to be examined during late infection by use of flow cytometry of infected organs and *in vivo* antibody depletion, as our data however, did not address this possibility.

In summary, MTb did not appear to activate or infect pDC *in vitro*. *In vivo*, no increase of plasmacytoid pDC was observed in lung and spleen following infection via the I.V. or aerosol route, and depletion of pDC during I.V. infection did not result in modulation of bacterial burdens in lung or spleen. Therefore, we conclude that plasmacytoid pDC do not play a role during the early phase of infection with MTb.

Chapter 6: General Discussion and Future Perspectives.

6.1. General discussion and future perspectives:

Immune homeostasis requires the harmonious interplay between specialised immune cells and their secreted soluble factors in order to regulate host inflammatory responses whilst promoting pathogen eradication without causing detrimental pathology. As observed in humans with naturally occurring mutations or gene deficient mice, the absence or an imbalance between any of these cells or their cytokines can have pathological or fatal consequences. Following infection, a pathogen may seek to survive and persist within a host by exploiting specific regulatory pathways normally associated with immune homeostasis. Similarly, the host may inadvertently promote pathogen persistence by limiting effector responses to invading organisms in order to limit damage to self. MTb could be described as a master of evasion by utilising a wide array of mechanisms for immune evasion in order to establish life-long latency in 90% of infected individuals. With this in mind, we sought to address the role of three potential regulatory pathways (IL-10, Tregs and plasmacytoid pDC), which may function either independently or in concert to promote persistence following infection with the bacterium MTb.

6.1.1. The role of IL-10 in regulating protective immunity to MTb infection:

The data we present in this thesis supports a role for IL-10 as a negative regulator of the immune response during both early and late stages of MTb infection. We demonstrate that during the early stages of infection IL-10 plays a role in regulating bacterial clearance that can be further enhanced in the absence of Tregs. This observation may highlight an important regulatory relationship on bacterial clearance between IL-10 and Tregs that has not been reported during infection with MTb.

During the latter stages of MTb infection, IL-10 is still present and plays a regulatory function to control cytokine production. Thus we postulate that IL-10's function switches from being a regulator of bacterial clearance to become a regulator of host immune-pathology in order to blunt over-exuberant effector responses.

The role of IL-10 in limiting detrimental host immune-pathology:

We have shown that IL-10 appears to be regulating cytokine production both during early and late MTb infection as demonstrated by *ex vivo* re-stimulations of cell suspensions from MTb infected mice. In contrast, using the approaches possible our data suggests that IL-10 only appears to be functioning to limit pathogen clearance during the early immune response to MTb. Therefore we postulate that IL-10 may be functioning during late MTb infection to regulate the immune response to limit host immune-pathology. In mouse models of *Toxoplasma gondii* (Gazzinelli *et al.*, 1996, Suzuki *et al.*, 2000), *Trypanosoma cruzi* (Hunter *et al.*, 1997) and malaria (Li *et al.*, 1999), in the absence of IL-10 parasite clearance is enhanced but leads to detrimental host immune-pathology and mortality. To date there is no published data on a role for IL-10 in limiting immune-pathology following either early or late/chronic MTb infection. We are currently addressing the role of IL-10 on immune-pathology during MTb infection by using histological analysis of infected organs such as the lung, spleen and liver. Examining the immune response in the absence of IL-10 at different stages of infection will provide insight into whether IL-10 affects the architecture of the granuloma including lymphocyte infiltration. We are also interested in determining other features of immune-pathology during MTb infection in the absence of IL-10, including weight loss.

Anti-IL-10R mAb as a potential adjuvant to enhance the efficacy of anti-mycobacterial chemotherapy:

We are currently examining whether treatment of mice with anti-IL10R mAb after the initiation of drug treatment during chronic MTb, may enhance bacterial killing and thus potentially speeding up the time to MTb clearance. The potential for using anti-IL-10R mAb during chemotherapy has already been demonstrated in murine visceral *Leishmaniasis*, as neutralisation of IL-10 activity during Pentostam chemotherapy, in order to lower parasite load, led to increased production of IFN-gamma and reduced time to parasite clearance (Murray *et al.*, 2002, Murray, 2005). Similarly, studies by Silva *et al.*, (2001) in *Mycobacterium avium* have also demonstrated a therapeutic role for anti-IL-10R mAb when administered at the time of chemotherapy, greatly enhancing its efficacy.

IL-10 mediated suppression of macrophage activation and MTb killing during in vitro infection:

Our studies have demonstrated that following *in vitro* MTb infection of BM macrophages, endogenous IL-10 appears to inhibit the induction of pro-inflammatory cytokines, namely IL-12p70, IL-12p40 and TNF (Fig. 3.11), but does not affect MTb killing (Fig. 3.12). With this in mind, we are currently determining whether in the absence of IL-10, IFN-gamma activation of macrophages (to mimic the T cell signal) enhances MTb killing. It has been shown by Shi *et al.*, (2003), that IFN-gamma activation of BM macrophages activates the expression of many genes encoding reactive oxygen and nitrogen intermediates and was dependent on the signalling molecule MyD88. However, in the same study it was also demonstrated

that macrophages activated via infection with live MTb also up-regulated genes to mediate bacterial killing that was independent of MyD88.

Macrophages and DC as sources for IL-10 during early MTb infection:

To address an *in vivo* role for macrophages and DC as sources of IL-10 during early MTb infection, we are currently breeding a Rag1, IL-10 double knockout mouse on the B6 background (B6.*Rag1*$^{-/-}$.*Il10*$^{-/-}$). Our rationale that macrophage-derived IL-10 may limit the response to MTb is based on our findings that the protection afforded by CD4+ T cells when adoptively transferred into BALB/c.*Rag2*$^{-/-}$ mice appeared to be independent of the action of T cell-derived IL-10 (Fig. 3.13). However in this Rag$^{-/-}$ transfer model, IL-10 could still have been coming a non-T cell source such as DC or macrophages; thus inhibiting any potential protective T cell immunity. Therefore we will repeat the adoptive transfers into B6.Rag$^{-/-}$.*Il10*$^{-/-}$ mice to address the role of non-T cell derived IL-10.

6.1.2. The potential role for naturally occurring Tregs in mediating MTb survival and persistence:

Despite observing a negative role for IL-10 in limiting anti-mycobacterial immunity, we could not isolate its cellular source. The advancement of reagents to study the role of Tregs during MTb infection has facilitated great interest in the role of this regulatory subset during chronic infections. We hypothesised that Tregs may regulate the immune response to MTb by production of IL-10 or other suppressive mediators as described in other experimental models such as colitis (Asseman *et al.*, 1999), *Leishmania* (Belkaid *et al.*, 2002) and *Helicobacter hepaticus* (Malloy *et al.*,

2003). The number of lung CD4+FoxP3+ Tregs increased significantly following aerosol challenge that suggested they maybe regulating the immune response early during infection. However, depletion of Tregs during MTb infection or adoptive transfer of Tregs into immuno-compromised hosts failed to confirm their regulatory role. As discussed earlier however, our data suggests that Tregs and IL-10 may function together in order to limit protective immunity during early MTb infection.

Tracking Treg activity in mice expressing a GFP tagged FoxP3:

We have shown in two independent experiments that CD4+FoxP3+ Tregs increase in the lung following aerosol MTb infection (Fig. 4.1 and Fig. 4.2). We have recently received a transgenic mouse expressing a GFP tagged FoxP3 (from B. Malissen). This transgenic mouse will enable us to as we performed previously to visualise the influx of FoxP3+ Tregs by flow cytometry, but in addition, we will also be able to sort an ultra-pure population of CD4+FoxP3+ Tregs for use in our adoptive transfer studies that may have greater regulatory potential since they will be completely devoid of any contaminating CD25+ effectors. Furthermore, the Tregs used in transfer models were purified on CD4+ and CD25+ expression alone thus eliminating a minor CD4+CD25-FoxP3+ Treg population.

Does adoptive transfer of Tregs into immuno-compromised mice regulate the early immune response to aerosol infection?

The adoptive transfer model used in our studies focused primarily on mice being infected by the I.V. route. This route of infection can be interpreted as a model for disseminated TB disease; therefore we intend to repeat our transfer studies using an aerosol infection model to mimic pulmonary TB.

6.1.3. The role of plasmacytoid pDC in anti-mycobacterial immunity:

To date, there is no direct evidence implicating a role for plasmacytoid pDC in regulating immunity to MTb infection, in contrast numerous reports have identified this DC subset as an essential player in the anti-viral response. In human studies, plasmacytoid pDC have been reported in the inflamed tonsil tissue of a TB patient (Cella *et al.*, 1999) and in the mouse the percentage of plasmacytoid pDC increases in the local draining lymph nodes in response to injection of heat-killed MTb (Blasius *et al.*, 2004). In our studies we demonstrate the plasmacytoid pDC were not infected with MTb or produced any pro-inflammatory cytokines in response to the bacterium. In addition, plasmacytoid pDC did not accumulate in MTb infected organs and depletion studies could not assign any regulatory role for this DC subset.

The role of plasmacytoid pDC following aerosol MTb infection:

Depletion studies during early I.V. infection revealed no apparent role for plasmacytoid pDC in regulating bacterial clearance. However, the study should be repeated using aerosol infection to address the role of plasmacytoid pDC during pulmonary TB.

The potential role for plasmacytoid pDC during the chronic stages of MTb infection:

During the latter stages of I.V. infection (Day 43) we observed a small increase in the absolute numbers of plasmacytoid pDC (Fig. 5.7). We hypothesise that during the latter stages of infection (or following antibiotic treatment), plasmacytoid pDC may become activated by pathogen related products e.g. non-methylated CpG-DNA,

that may be released into the local milieu by dead bacteria. Therefore we would like to assess the influx of plasmacytoid pDC to the lung and draining lymph nodes by flow cytometry during the course of MTb infection and in the presence of antibiotics. In addition, 120G8 mAb depletion of plasmacytoid pDC late after MTb infection will allow us to confirm if this DC subset plays any regulatory role as suggested previously in models of allergy (de Heer *et al.*, 2004) and graft tolerance (Orchando *et al.*, 2006).

In summary, our studies suggest that IL-10 and Tregs both function together in order to regulate bacterial clearance during the immune response to MTb. In contrast to murine studies in asthma, we could assign no regulatory role for plasmacytoid pDC on bacterial clearance. Our findings highlight an important regulatory pathway that MTb could be exploiting in order to survive and persist within infected hosts that may have important clinical implications for the design of novel immune therapies.

Chapter 7: References:

Abel, B., Thieblemont, N., Quesniaux, V. J., Brown, N., Mpagi, J., Miyake, K., Bihl, F. and Ryffel, B. (2002), Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice, *J Immunol,* **169,** 3155-3162.

Akira, S. and Takeda, K. (2004), Functions of toll-like receptors: lessons from KO mice, *C R Biol,* **327,** 581-589.

Allen, S. S., Cassone, L., Lasco, T. M. and McMurray, D. N. (2004), Effect of neutralizing transforming growth factor beta1 on the immune response against Mycobacterium tuberculosis in guinea pigs, *Infect Immun,* **72,** 1358-1363.

Altare, F., Durandy, A., Lammas, D., Emile, J. F., Lamhamedi, S., Le Deist, F., Drysdale, P., Jouanguy, E., Doffinger, R., Bernaudin, F., Jeppsson, O., Gollob, J. A., Meinl, E., Segal, A. W., Fischer, A., Kumararatne, D. and Casanova, J. L. (1998), Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency, *Science,* **280,** 1432-1435.

Andersen, P. and Doherty, T. M. (2005), The success and failure of BCG - implications for a novel tuberculosis vaccine, *Nat Rev Microbiol,* **3,** 656-662.

Anderson, C. F., Mendez, S. and Sacks, D. L. (2005), Nonhealing infection despite Th1 polarization produced by a strain of Leishmania major in C57BL/6 mice, *J Immunol,* **174,** 2934-2941.

Andersson, J., Boasso, A., Nilsson, J., Zhang, R., Shire, N. J., Lindback, S., Shearer, G. M. and Chougnet, C. A. (2005), The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients, *J Immunol,* **174,** 3143-3147.

Armstrong, J. A. and Hart, P. D. (1971), Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes, *J Exp Med,* **134,** 713-740.

Armstrong, J. A. and Hart, P. D. (1975), Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival, *J Exp Med,* **142,** 1-16.

Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F. and Trinchieri, G. (2001), Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology, *Nat Immunol,* **2,** 1144-1150.

Asseman, C., Mauze, S., Leach, M.W., Coffman, R.L., and Powrie, F. (1999), An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation, *J. Exp. Med.,* **190,** 995-1003.

Asseman, C., Read, S. and Powrie, F. (2003), Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4+ regulatory T cells and IL-10, *J Immunol,* **171,** 971-978.

Auerbuch, V., Brockstedt, D. G., Meyer-Morse, N., O'Riordan, M. and Portnoy, D. A. (2004), Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes, *J Exp Med,* **200,** 527-533.

Bafica, A., Scanga, C. A., Feng, C. G., Leifer, C., Cheever, A. and Sher, A. (2005), TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis, *J Exp Med,* **202,** 1715-1724.

Balkhi, M. Y., Sinha, A. and Natarajan, K. (2004), Dominance of CD86, transforming growth factor- beta 1, and interleukin-10 in Mycobacterium

tuberculosis secretory antigen-activated dendritic cells regulates T helper 1 responses to mycobacterial antigens, *J Infect Dis,* **189,** 1598-1609.

Banchereau, J., and Steinman, R.M. (1998), Dendritic cells and the control of immunity, *Nature,* **392,** 245-252.

Barchet, W., Cella, M., Odermatt, B., Asselin-Paturel, C., Colonna, M. and Kalinke, U. (2002), Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling in vivo, *J Exp Med,* **195,** 507-516.

Barrat, F. J., Cua, D. J., Boonstra, A., Richards, D. F., Crain, C., Savelkoul, H. F., de Waal-Malefyt, R., Coffman, R. L., Hawrylowicz, C. M. and O'Garra, A. (2002), In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines, *J Exp Med,* **195,** 603-616.

Baumann, S., Eddine, A. N. and Kaufmann, S. H. (2006), Progress in tuberculosis vaccine development, *Curr Opin Immunol,* **18,** 438-448.

Bean, A. G., Roach, D. R., Briscoe, H., France, M. P., Korner, H., Sedgwick, J. D. and Britton, W. J. (1999), Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin, *J Immunol,* **162,** 3504-3511.

Belkaid, Y., Hoffmann, K. F., Mendez, S., Kamhawi, S., Udey, M. C., Wynn, T. A. and Sacks, D. L. (2001), The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure, *J Exp Med,* **194,** 1497-1506.

Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M. and Sacks, D. L. (2002), CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity, *Nature,* **420,** 502-507.

Belkaid, Y. and Rouse, B. T. (2005), Natural regulatory T cells in infectious disease, *Nat Immunol,* **6,** 353-360.

Bermudez, L. E. and Champsi, J. (1993), Infection with Mycobacterium avium induces production of interleukin-10 (IL-10), and administration of anti-IL-10 antibody is associated with enhanced resistance to infection in mice, *Infect Immun,* **61,** 3093-3097.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L. and Kuchroo, V. K. (2006), Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells, *Nature,* **441,** 235-238.

Bjorck, P. (2001), Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice, *Blood,* **98,** 3520-3526.

Blasius, A., Vermi, W., Krug, A., Facchetti, F., Cella, M. and Colonna, M. (2004), A cell-surface molecule selectively expressed on murine natural interferon-producing cells that blocks secretion of interferon-alpha, *Blood,* **103,** 4201-4206.

Bodnar, K. A., Serbina, N. V. and Flynn, J. L. (2001), Fate of Mycobacterium tuberculosis within murine dendritic cells, *Infect Immun,* **69,** 800-809.

Boonstra, A., Asselin-Paturel, C., Gilliet, M., Crain, C., Trinchieri, G., Liu, Y. J. and O'Garra, A. (2003), Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development:

dependency on antigen dose and differential toll-like receptor ligation, *J Exp Med,* **197,** 101-109.

Boonstra, A., Rajsbaum, R., Holman, M., Marques, R., Asselin-Paturel, C., Pereira, J. P., Bates, E. E. M., Akira, S., Vieira, P., Liu, Y.-J., Trinchieri, G. and O'Garra, A. (2006), Macrophages and Myeloid Dendritic Cells, but Not Plasmacytoid Dendritic Cells, Produce IL-10 in Response to MyD88- and TRIF-Dependent TLR Signals, and TLR-Independent Signals, *J Immunol,* **177,** 7551-7558.

Bosio, C. M., Gardner, D. and Elkins, K. L. (2000), Infection of B cell-deficient mice with CDC 1551, a clinical isolate of Mycobacterium tuberculosis: delay in dissemination and development of lung pathology, *J Immunol,* **164,** 6417-6425.

Boussiotis, V. A., Tsai, E. Y., Yunis, E. J., Thim, S., Delgado, J. C., Dascher, C. C., Berezovskaya, A., Rousset, D., Reynes, J. M. and Goldfeld, A. E. (2000), IL-10-producing T cells suppress immune responses in anergic tuberculosis patients, *J Clin Invest,* **105,** 1317-1325.

Brigl, M. and Brenner, M. B. (2004), CD1: antigen presentation and T cell function, *Annu Rev Immunol,* **22,** 817-890.

Buer, J., Lanoue, A., Franzke, A., Garcia, C., von Boehmer, H., and Sarukhan, A. (1998), Interleukin 10 secretion and impaired effector function of major histocmpatibility complex class II-restricted T cells anergized in vivo, *J. Exp. Med.,* **187,** 177-183.

Burkhart, C., Liu, G.Y., Anderton, S.M., Metzler, B., Wraith, D.C. (1999), Peptide-induced T cell regulation of experimental autoimmune encephalomyelitis: a role for IL-10, *Internat. Immunol.,* **11,** 1625-1634.

Campanelli, A. P., Roselino, A. M., Cavassani, K. A., Pereira, M. S., Mortara, R. A., Brodskyn, C. I., Goncalves, H. S., Belkaid, Y., Barral-Netto, M., Barral, A. and Silva, J. S. (2006), CD4+CD25+ T cells in skin lesions of patients with cutaneous leishmaniasis exhibit phenotypic and functional characteristics of natural regulatory T cells, *J Infect Dis,* **193,** 1313-1322.

Carrero, J. A., Calderon, B. and Unanue, E. R. (2004), Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection, *J Exp Med,* **200,** 535-540.

Carrero, J. A., Calderon, B. and Unanue, E. R. (2006), Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes, *J Exp Med,* **203,** 933-940.

Caruso, A. M., Serbina, N., Klein, E., Triebold, K., Bloom, B. R. and Flynn, J. L. (1999), Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis, *J Immunol,* **162,** 5407-5416.

Casanova, J. L. and Abel, L. (2002), Genetic dissection of immunity to mycobacteria: the human model, *Annu Rev Immunol,* **20,** 581-620.

Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A. and Colonna, M. (1999), Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon, *Nat Med,* **5,** 919-923.

Chan, J., Tanaka, K., Carroll, D., Flynn, J. and Bloom, B. R. (1995), Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis, *Infect Immun,* **63,** 736-740.

Chan, J., Xing, Y., Magliozzo, R. S. and Bloom, B. R. (1992), Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages, *J Exp Med,* **175,** 1111-1122.

Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G. and Wahl, S. M. (2003), Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3, *J Exp Med,* **198,** 1875-1886.

Co, D. O., Hogan, L. H., Kim, S. I. and Sandor, M. (2004), Mycobacterial granulomas: keys to a long-lasting host-pathogen relationship, *Clin Immunol,* **113,** 130-136.

Collins, H. L., Kaufmann, S. H. and Schaible, U. E. (2002), Iron chelation via deferoxamine exacerbates experimental salmonellosis via inhibition of the nicotinamide adenine dinucleotide phosphate oxidase-dependent respiratory burst, *J Immunol,* **168,** 3458-3463.

Converse, P. J., Dannenberg, A. M., Jr., Estep, J. E., Sugisaki, K., Abe, Y., Schofield, B. H. and Pitt, M. L. (1996), Cavitary tuberculosis produced in rabbits by aerosolized virulent tubercle bacilli, *Infect Immun,* **64,** 4776-4787.

Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G. and Orme, I. M. (1993), Disseminated tuberculosis in interferon gamma gene-disrupted mice, *J Exp Med,* **178,** 2243-2247.

Cooper, A. M., Kipnis, A., Turner, J., Magram, J., Ferrante, J. and Orme, I. M. (2002), Mice lacking bioactive IL-12 can generate protective, antigen-specific cellular responses to mycobacterial infection only if the IL-12 p40 subunit is present, *J Immunol,* **168,** 1322-1327.

Cooper, A. M., Magram, J., Ferrante, J. and Orme, I. M. (1997), Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis, *J Exp Med,* **186,** 39-45.

Cooper, A. M., Roberts, A. D., Rhoades, E. R., Callahan, J. E., Getzy, D. M. and Orme, I. M. (1995), The role of interleukin-12 in acquired immunity to Mycobacterium tuberculosis infection, *Immunology,* **84,** 423-432.

Cousens, L. P., Orange, J. S., Su, H. C. and Biron, C. A. (1997), Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection, *Proc Natl Acad Sci U S A,* **94,** 634-639.

Cruz, A., Khader, S. A., Torrado, E., Fraga, A., Pearl, J. E., Pedrosa, J., Cooper, A. M. and Castro, A. G. (2006), Cutting edge: IFN-gamma regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection, *J Immunol,* **177,** 1416-1420.

D'Souza, C. D., Cooper, A. M., Frank, A. A., Mazzaccaro, R. J., Bloom, B. R. and Orme, I. M. (1997), An anti-inflammatory role for gamma delta T lymphocytes in acquired immunity to Mycobacterium tuberculosis, *J Immunol,* **158,** 1217-1221.

Dai, W. J., Kohler, G., and Brombacher, F. (1997), Both innate and acquired immune immunity to *Listeria monocytogenes* infection are increased in IL-10-deficient mice, *J. Immunol.,* **158,** 2259-2267.

Dalod, M., Hamilton, T., Salomon, R., Salazar-Mather, T. P., Henry, S. C., Hamilton, J. D. and Biron, C. A. (2003), Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta, *J Exp Med,* **197,** 885-898.

de Heer, H. J., Hammad, H., Soullie, T., Hijdra, D., Vos, N., Willart, M. A., Hoogsteden, H. C. and Lambrecht, B. N. (2004), Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen, *J Exp Med,* **200,** 89-98.

de Jong, R., Altare, F., Haagen, I. A., Elferink, D. G., Boer, T., van Breda Vriesman, P. J., Kabel, P. J., Draaisma, J. M., van Dissel, J. T., Kroon, F. P., Casanova, J. L. and Ottenhoff, T. H. (1998), Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients, *Science,* **280,** 1435-1438.

De Libero, G. and Mori, L. (2005), Recognition of lipid antigens by T cells, *Nat Rev Immunol,* **5,** 485-496.

Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S. and Reis e Sousa, C. (2004), Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA, *Science,* **303,** 1529-1531.

Dillon, S., Agrawal, A., Van Dyke, T., Landreth, G., McCauley, L., Koh, A., Maliszewski, C., Akira, S. and Pulendran, B. (2004), A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells, *J Immunol,* **172,** 4733-4743.

Dorman, S. E. and Holland, S. M. (2000), Interferon-gamma and interleukin-12 pathway defects and human disease, *Cytokine Growth Factor Rev,* **11,** 321-333.

Dorman, S. E., Picard, C., Lammas, D., Heyne, K., van Dissel, J. T., Baretto, R., Rosenzweig, S. D., Newport, M., Levin, M., Roesler, J., Kumararatne, D., Casanova, J. L. and Holland, S. M. (2004), Clinical features of dominant and recessive interferon gamma receptor 1 deficiencies, *Lancet,* **364,** 2113-2121.

Engwerda, C. R., Ato, M. and Kaye, P. M. (2004), Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis, *Trends Parasitol,* **20,** 524-530.

Fahlen, L., Read, S., Gorelik, L., Hurst, S. D., Coffman, R. L., Flavell, R. A. and Powrie, F. (2005), T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells, *J Exp Med,* **201,** 737-746.

Fantini, M. C., Becker, C., Monteleone, G., Pallone, F., Galle, P. R. and Neurath, M. F. (2004), Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7, *J Immunol,* **172,** 5149-5153.

Fayyazi, A., Eichmeyer, B., Soruri, A., Schweyer, S., Herms, J., Schwarz, P. and Radzun, H. J. (2000), Apoptosis of macrophages and T cells in tuberculosis associated caseous necrosis, *J Pathol,* **191,** 417-425.

Feng, C. G., Bean, A. G., Hooi, H., Briscoe, H. and Britton, W. J. (1999), Increase in gamma interferon-secreting CD8(+), as well as CD4(+), T cells in lungs following aerosol infection with Mycobacterium tuberculosis, *Infect Immun,* **67,** 3242-3247.

Feng, C. G., Kullberg, M. C., Jankovic, D., Cheever, A. W., Caspar, P., Coffman, R. L. and Sher, A. (2002), Transgenic mice expressing human interleukin-10 in the antigen-presenting cell compartment show increased susceptibility to infection with Mycobacterium avium associated with decreased macrophage effector function and apoptosis, *Infect Immun,* **70,** 6672-6679.

Fenhalls, G., Stevens, L., Bezuidenhout, J., Amphlett, G. E., Duncan, K., Bardin, P. and Lukey, P. T. (2002), Distribution of IFN-gamma, IL-4 and TNF-alpha protein and CD8 T cells producing IL-12p40 mRNA in human lung tuberculous granulomas, *Immunology,* **105,** 325-335.

Fenhalls, G., Wong, A., Bezuidenhout, J., van Helden, P., Bardin, P. and Lukey, P. T. (2000), In situ production of gamma interferon, interleukin-4, and tumor necrosis factor alpha mRNA in human lung tuberculous granulomas, *Infect Immun,* **68,** 2827-2836.

Fine, P. E. (1995), Variation in protection by BCG: implications of and for heterologous immunity, *Lancet,* **346,** 1339-1345.

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. and O'Garra, A. (1991a), IL-10 inhibits cytokine production by activated macrophages, *J. Immunol.,* **147,** 3815-3822.

Fiorentino, D. F., Zlotnik, A., Vieira, P., Mosmann, T. R., Howard, M., Moore, K. W. and O'Garra, A. (1991b), IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells, *J.Immunol.,* **146,** 3444-3451.

Fiorenza, G., Rateni, L., Farroni, M. A., Bogue, C. and Dlugovitzky, D. G. (2005), TNF-alpha, TGF-beta and NO relationship in sera from tuberculosis (TB) patients of different severity, *Immunol Lett,* **98,** 45-48.

Flores-Villanueva, P. O., Ruiz-Morales, J. A., Song, C. H., Flores, L. M., Jo, E. K., Montano, M., Barnes, P. F., Selman, M. and Granados, J. (2005), A functional promoter polymorphism in monocyte chemoattractant protein-1 is associated with increased susceptibility to pulmonary tuberculosis, *J Exp Med,* **202,** 1649-1658.

Flynn, J. L. and Chan, J. (2001), Immunology of tuberculosis, *Annu Rev Immunol,* **19,** 93-129.

Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A. and Bloom, B. R. (1993), An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection, *J Exp Med,* **178,** 2249-2254.

Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., Schreiber, R., Mak, T. W. and Bloom, B. R. (1995a), Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice, *Immunity,* **2,** 561-572.

Flynn, J. L., Goldstein, M. M., Triebold, K. J., Koller, B. and Bloom, B. R. (1992), Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection, *Proc Natl Acad Sci U S A,* **89,** 12013-12017.

Flynn, J. L., Goldstein, M. M., Triebold, K. J., Sypek, J., Wolf, S. and Bloom, B. R. (1995b), IL-12 increases resistance of BALB/c mice to Mycobacterium tuberculosis infection, *J Immunol,* **155,** 2515-2524.

Flynn, J. L., Scanga, C. A., Tanaka, K. E. and Chan, J. (1998), Effects of aminoguanidine on latent murine tuberculosis, *J Immunol,* **160,** 1796-1803.

Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y. (2003), Foxp3 programs the development and function of CD4+CD25+ regulatory T cells, *Nat Immunol,* **4,** 330-336.

Fontenot, J. D., Rasmussen, J. P., Williams, L. M., Dooley, J. L., Farr, A. G. and Rudensky, A. Y. (2005a), Regulatory T cell lineage specification by the forkhead transcription factor foxp3, *Immunity,* **22,** 329-341.

Fontenot, J. D. and Rudensky, A. Y. (2005b), A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3, *Nat Immunol,* **6,** 331-337.

Frieden, T. R., Sterling, T. R., Munsiff, S. S., Watt, C. J. and Dye, C. (2003), Tuberculosis, *Lancet,* **362,** 887-899.

Gagliardi, M. C., Teloni, R., Giannoni, F., Pardini, M., Sargentini, V., Brunori, L., Fattorini, L. and Nisini, R. (2005), Mycobacterium bovis Bacillus Calmette-Guerin infects DC-SIGN- dendritic cell and causes the inhibition of IL-12 and the enhancement of IL-10 production, *J Leukoc Biol,* **78,** 106-113.

Garcia-Romo, G. S., Pedroza-Gonzalez, A., Aguilar-Leon, D., Orozco-Estevez, H., Lambrecht, B. N., Estrada-Garcia, I., Flores-Romo, L. and Hernandez-Pando, R. (2004), Airways infection with virulent Mycobacterium tuberculosis delays the influx of dendritic cells and the expression of costimulatory molecules in mediastinal lymph nodes, *Immunology,* **112,** 661-668.

Gautier, G., Humbert, M., Deauvieau, F., Scuiller, M., Hiscott, J., Bates, E. E., Trinchieri, G., Caux, C. and Garrone, P. (2005), A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells, *J Exp Med,* **201,** 1435-1446.

Gazzinelli, R. T., Oswald, I. P., James, S. L. and Sher, A. (1992), IL-10 inhibits parasite killing and nitric oxide production by IFN-γ activated macrophages, *J. Immunol.,* **148,** 1792-1796.

Gazzinelli, R. T., Wysocka, M., Hieny, S., Scharton-Kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G. and Sher, A. (1996), In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha, *J Immunol,* **157,** 798-805.

Gerosa, F., Nisii, C., Righetti, S., Micciolo, R., Marchesini, M., Cazzadori, A. and Trinchieri, G. (1999), CD4(+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients, *Clin Immunol,* **92,** 224-234.

Giacomini, E., Iona, E., Ferroni, L., Miettinen, M., Fattorini, L., Orefici, G., Julkunen, I. and Coccia, E. M. (2001), Infection of human macrophages and dendritic cells with Mycobacterium tuberculosis induces a differential cytokine gene expression that modulates T cell response, *J Immunol,* **166,** 7033-7041.

Gomes, M. S., Florido, M., Pais, T. F. and Appelberg, R. (1999), Improved clearance of Mycobacterium avium upon disruption of the inducible nitric oxide synthase gene, *J Immunol,* **162,** 6734-6739.

Gong, J. H., Zhang, M., Modlin, R. L., Linsley, P. S., Iyer, D., Lin, Y. and Barnes, P. F. (1996), Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression, *Infect Immun,* **64,** 913-918.

Gonzalez-Juarrero, M. and Orme, I. M. (2001a), Characterization of murine lung dendritic cells infected with Mycobacterium tuberculosis, *Infect Immun,* **69,** 1127-1133.

Gonzalez-Juarrero, M., Shim, T. S., Kipnis, A., Junqueira-Kipnis, A. P. and Orme, I. M. (2003), Dynamics of macrophage cell populations during murine pulmonary tuberculosis, *J Immunol,* **171,** 3128-3135.

Gonzalez-Juarrero, M., Turner, O. C., Turner, J., Marietta, P., Brooks, J. V. and Orme, I. M. (2001b), Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with Mycobacterium tuberculosis, *Infect Immun,* **69,** 1722-1728.

Grode, L., Seiler, P., Baumann, S., Hess, J., Brinkmann, V., Nasser Eddine, A., Mann, P., Goosmann, C., Bandermann, S., Smith, D., Bancroft, G. J., Reyrat, J. M., van Soolingen, D., Raupach, B. and Kaufmann, S. H. (2005), Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin, *J Clin Invest,* **115,** 2472-2479.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. and Roncarolo, M. G. (1997), A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis, *Nature,* **389,** 737-742.

Guyot-Revol, V., Innes, J. A., Hackforth, S., Hinks, T. and Lalvani, A. (2006), Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis, *Am J Respir Crit Care Med,* **173,** 803-810.

Harboe, M., Oettinger, T., Wiker, H. G., Rosenkrands, I. and Andersen, P. (1996), Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG, *Infect Immun,* **64,** 16-22.

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M. and Weaver, C. T. (2005), Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages, *Nat Immunol,* **6,** 1123-1132.

Harrington, L. E., Mangan, P. R. and Weaver, C. T. (2006), Expanding the effector CD4 T-cell repertoire: the Th17 lineage, *Curr Opin Immunol,* **18,** 349-356.

Hawrylowicz, C. M. (2005), Regulatory T cells and IL-10 in allergic inflammation, *J Exp Med,* **202,** 1459-1463.

Hawrylowicz, C. M. and O'Garra, A. (2005), Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma, *Nat Rev Immunol,* **5,** 271-283.

Henderson, R. A., Watkins, S. C. and Flynn, J. L. (1997), Activation of human dendritic cells following infection with Mycobacterium tuberculosis, *J Immunol,* **159,** 635-643.

Hernandez-Garay, M. and Mendez-Samperio, P. (2003), Transforming growth factor-beta decreases survival of Mycobacterium bovis-activated T cells, *Arch Med Res,* **34,** 20-25.

Hernandez-Pando, R., Orozcoe, H., Sampieri, A., Pavon, L., Velasquillo, C., Larriva-Sahd, J., Alcocer, J. M. and Madrid, M. V. (1996), Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis, *Immunology,* **89,** 26-33.

Hickman, S. P., Chan, J. and Salgame, P. (2002), Mycobacterium tuberculosis induces differential cytokine production from dendritic cells and macrophages with divergent effects on naive T cell polarization, *J Immunol,* **168,** 4636-4642.

Hisaeda, H., Maekawa, Y., Iwakawa, D., Okada, H., Himeno, K., Kishihara, K., Tsukumo, S. and Yasutomo, K. (2004), Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells, *Nat Med,* **10,** 29-30.

Hori, S., Nomura, T. and Sakaguchi, S. (2003), Control of regulatory T cell development by the transcription factor Foxp3, *Science,* **299,** 1057-1061.

Howard, A. D. and Zwilling, B. S. (1999), Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines, *Clin Exp Immunol,* **115,** 428-434.

HPA - UK (2006). http://www.hpa.org.uk/infections/topics_az/tb/data_menu.htm

Humphreys, I. R., Stewart, G. R., Turner, D. J., Patel, J., Karamanou, D., Snelgrove, R. J. and Young, D. B. (2006), A role for dendritic cells in the dissemination of mycobacterial infection, *Microbes Infect,* **8,** 1339-1346.

Hunter, C. A., Ellis-Neyes, L. A., Slifer, T., Kanaly, S., Grunig, G., Fort, M., Rennick, D. and Araujo, F. G. (1997), IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi, *J Immunol,* **158,** 3311-3316.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S. and Steinman, R. M. (1992), Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor, *J. Exp. Med.*, **176,** 1693.

Izaguirre, A., Barnes, B. J., Amrute, S., Yeow, W. S., Megjugorac, N., Dai, J., Feng, D., Chung, E., Pitha, P. M. and Fitzgerald-Bocarsly, P. (2003), Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells, *J Leukoc Biol,* **74,** 1125-1138.

Jouanguy, E., Altare, F., Lamhamedi, S., Revy, P., Emile, J. F., Newport, M., Levin, M., Blanche, S., Seboun, E., Fischer, A. and Casanova, J. L. (1996), Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guerin infection, *N Engl J Med,* **335,** 1956-1961.

Jung, Y. J., Ryan, L., LaCourse, R. and North, R. J. (2003), Increased interleukin-10 expression is not responsible for failure of T helper 1 immunity to resolve airborne Mycobacterium tuberculosis infection in mice, *Immunology,* **109,** 295-299.

Junqueira-Kipnis, A. P., Turner, J., Gonzalez-Juarrero, M., Turner, O. C. and Orme, I. M. (2004), Stable T-cell population expressing an effector cell surface phenotype in the lungs of mice chronically infected with Mycobacterium tuberculosis, *Infect Immun,* **72,** 570-575.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F. and Liu, Y. J. (2001), Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens, *J Exp Med,* **194,** 863-869.

Kapsenberg, M. L. (2003), Dendritic-cell control of pathogen-driven T-cell polarization, *Nat Rev Immunol,* **3,** 984-993.

Kaufmann, S. H. (2001), How can immunology contribute to the control of tuberculosis?, *Nat Rev Immunol,* **1,** 20-30.

Kaufmann, S. H. (2006a), Envisioning future strategies for vaccination against tuberculosis, *Nat Rev Immunol,* **6,** 699-704.

Kaufmann, S. H. (2006b), Tuberculosis: back on the immunologists' agenda, *Immunity,* **24,** 351-357.

Keane, J., Gershon, S., Wise, R. P., Mirabile-Levens, E., Kasznica, J., Schwieterman, W. D., Siegel, J. N. and Braun, M. M. (2001), Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent, *N Engl J Med,* **345,** 1098-1104.

Kearley, J., Barker, J. E., Robinson, D. S. and Lloyd, C. M. (2005), Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent, *J Exp Med,* **202,** 1539-1547.

Khader, S. A., Partida-Sanchez, S., Bell, G., Jelley-Gibbs, D. M., Swain, S., Pearl, J. E., Ghilardi, N., Desauvage, F. J., Lund, F. E. and Cooper, A. M. (2006), Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection, *J Exp Med,* **203,** 1805-1815.

Khader, S. A., Pearl, J. E., Sakamoto, K., Gilmartin, L., Bell, G. K., Jelley-Gibbs, D. M., Ghilardi, N., deSauvage, F. and Cooper, A. M. (2005), IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-gamma responses if IL-12p70 is available, *J Immunol,* **175,** 788-795.

Khattri, R., Cox, T., Yasayko, S. A. and Ramsdell, F. (2003), An essential role for Scurfin in CD4+CD25+ T regulatory cells, *Nat Immunol,* **4,** 337-342.

Krug, A., Luker, G. D., Barchet, W., Leib, D. A., Akira, S. and Colonna, M. (2004), Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9, *Blood,* **103,** 1433-1437.

Kursar, M., Bonhagen, K., Fensterle, J., Kohler, A., Hurwitz, R., Kamradt, T., Kaufmann, S. H. and Mittrucker, H. W. (2002), Regulatory CD4+CD25+ T cells restrict memory CD8+ T cell responses, *J Exp Med,* **196,** 1585-1592.

Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A. and Cua, D. J. (2005), IL-23 drives a pathogenic T cell population that induces autoimmune inflammation, *J Exp Med,* **201,** 233-240.

Lanzavecchia, A. and Sallusto, F. (2001), Regulation of T cell immunity by dendritic cells, *Cell,* **106,** 263-266.

Laouar, Y., Sutterwala, F. S., Gorelik, L. and Flavell, R. A. (2005), Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma, *Nat Immunol,* **6,** 600-607.

Lazarevic, V., Nolt, D. and Flynn, J. L. (2005), Long-term control of Mycobacterium tuberculosis infection is mediated by dynamic immune responses, *J Immunol,* **175,** 1107-1117.

Letterio, J. J. and Roberts, A. B. (1997), TGF-beta: a critical modulator of immune cell function, *Clin Immunol Immunopathol,* **84,** 244-250.

Levings, M. K., Sangregorio, R. and Roncarolo, M. G. (2001), Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function, *J Exp Med,* **193,** 1295-1302.

Li, C., Corraliza, I. and Langhorne, J. (1999), A defect in interleukin-10 leads to enhanced malarial disease in Plasmodium chabaudi chabaudi infection in mice, *Infect Immun,* **67,** 4435-4442.

Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A.-K. L. and Flavell, R. A. (2006a), Transforming Growth Factor-beta regulation of immune responses, *Annual Review of Immunology,* **24,** 99-146.

Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K. and Flavell, R. A. (2006b), Transforming growth factor-beta regulation of immune responses, *Annu Rev Immunol,* **24,** 99-146.

Lichtner, M., Rossi, R., Mengoni, F., Vignoli, S., Colacchia, B., Massetti, A. P., Kamga, I., Hosmalin, A., Vullo, V. and Mastroianni, C. M. (2006), Circulating dendritic cells and interferon-alpha production in patients with tuberculosis: correlation with clinical outcome and treatment response, *Clin Exp Immunol,* **143,** 329-337.

Liu, H., Hu, B., Xu, D. and Liew, F. Y. (2003), CD4+CD25+ regulatory T cells cure murine colitis: the role of IL-10, TGF-beta, and CTLA4, *J Immunol,* **171,** 5012-5017.

Liu, Y. J. (2005), IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors, *Annu Rev Immunol,* **23,** 275-306.

Lockhart, E., Green, A. M. and Flynn, J. L. (2006), IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection, *J Immunol,* **177,** 4662-4669.

Lund, J. M., Alexopoulou, L., Sato, A., Karow, M., Adams, N. C., Gale, N. W., Iwasaki, A. and Flavell, R. A. (2004), Recognition of single-stranded RNA viruses by Toll-like receptor 7, *Proc Natl Acad Sci U S A,* **101,** 5598-5603.

MacMicking, J. D., North, R. J., LaCourse, R., Mudgett, J. S., Shah, S. K. and Nathan, C. F. (1997), Identification of nitric oxide synthase as a protective locus against tuberculosis, *Proc Natl Acad Sci U S A,* **94,** 5243-5248.

Maloy, K. J., Salaun, L., Cahill, R., Dougan, G., Saunders, N. J. and Powrie, F. (2003), CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms, *J Exp Med,* **197,** 111-119.

Manca, C., Tsenova, L., Bergtold, A., Freeman, S., Tovey, M., Musser, J. M., Barry, C. E., 3rd, Freedman, V. H. and Kaplan, G. (2001), Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta, *Proc Natl Acad Sci U S A,* **98,** 5752-5757.

Manca, C., Tsenova, L., Freeman, S., Barczak, A. K., Tovey, M., Murray, P. J., Barry, C. and Kaplan, G. (2005), Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway, *J Interferon Cytokine Res,* **25,** 694-701.

Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R. and Weaver, C. T. (2006), Transforming growth factor-beta induces development of the T(H)17 lineage, *Nature,* **441,** 231-234.

Marie, J. C., Letterio, J. J., Gavin, M. and Rudensky, A. Y. (2005), TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells, *J Exp Med,* **201,** 1061-1067.

Marshall, B. G., Chambers, M. A., Wangoo, A., Shaw, R. J. and Young, D. B. (1997), Production of tumor necrosis factor and nitric oxide by macrophages infected with live and dead mycobacteria and their suppression by an interleukin-10-secreting recombinant, *Infect Immun,* **65,** 1931-1935.

McKee, A. S. and Pearce, E. J. (2004), CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development, *J Immunol,* **173,** 1224-1231.

McKinney, J. D., Honer zu Bentrup, K., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr. and Russell, D. G. (2000), Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase, *Nature,* **406,** 735-738.

McMurray, D. N. (2001), Disease model: pulmonary tuberculosis, *Trends Mol Med,* **7,** 135-137.

Means, T. K., Jones, B. W., Schromm, A. B., Shurtleff, B. A., Smith, J. A., Keane, J., Golenbock, D. T., Vogel, S. N. and Fenton, M. J. (2001), Differential effects of a Toll-like receptor antagonist on Mycobacterium tuberculosis-induced macrophage responses, *J Immunol,* **166,** 4074-4082.

Means, T. K., Wang, S., Lien, E., Yoshimura, A., Golenbock, D. T. and Fenton, M. J. (1999), Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis, *J Immunol,* **163,** 3920-3927.

Medzhitov, R. (2001), Toll-like receptors and innate immunity, *Nat Rev Immunol,* **1,** 135-145.

Mendez, S., Reckling, S. K., Piccirillo, C. A., Sacks, D. and Belkaid, Y. (2004), Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity, *J Exp Med,* **200,** 201-210.

Mills, K. H. (2004), Regulatory T cells: friend or foe in immunity to infection?, *Nat Rev Immunol,* **4,** 841-855.

Mogga, S. J., Mustafa, T., Sviland, L. and Nilsen, R. (2003), In situ expression of CD40, CD40L (CD154), IL-12, TNF-alpha, IFN-gamma and TGF-beta1 in murine lungs during slowly progressive primary tuberculosis, *Scand J Immunol,* **58,** 327-334.

Mogues, T., Goodrich, M. E., Ryan, L., LaCourse, R. and North, R. J. (2001), The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice, *J Exp Med,* **193,** 271-280.

Mohan, V. P., Scanga, C. A., Yu, K., Scott, H. M., Tanaka, K. E., Tsang, E., Tsai, M. M., Flynn, J. L. and Chan, J. (2001), Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology, *Infect Immun,* **69,** 1847-1855.

Moody, D. B., Young, D. C., Cheng, T. Y., Rosat, J. P., Roura-Mir, C., O'Connor, P. B., Zajonc, D. M., Walz, A., Miller, M. J., Levery, S. B., Wilson, I. A., Costello, C. E. and Brenner, M. B. (2004), T cell activation by lipopeptide antigens, *Science,* **303,** 527-531.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. and O'Garra, A. (2001), Interleukin-10 and the interleukin-10 receptor, *Annu Rev Immunol,* **19,** 683-765.

Murray, H. W. (2005), Interleukin 10 receptor blockade--pentavalent antimony treatment in experimental visceral leishmaniasis, *Acta Trop,* **93,** 295-301.

Murray, H. W., Lu, C. M., Mauze, S., Freeman, S., Moreira, A. L., Kaplan, G. and Coffman, R. L. (2002), Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy, *Infect Immun,* **70,** 6284-6293.

Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L. P., Allavena, P. and Mantovani, A. (2000), Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells, *J Immunol,* **164,** 5998-6004.

Nakano, H., Yanagita, M. and Gunn, M. D. (2001), CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells, *J Exp Med,* **194,** 1171-1178.

Newport, M. J., Awomoyi, A. A. and Blackwell, J. M. (2003), Polymorphism in the interferon-gamma receptor-1 gene and susceptibility to pulmonary tuberculosis in The Gambia, *Scand J Immunol,* **58,** 383-385.

North, R. J. (1998), Mice incapable of making IL-4 or IL-10 display normal resistance to infection with Mycobacterium tuberculosis, *Clin Exp Immunol,* **113,** 55-58.

North, R. J. and Jung, Y. J. (2004), Immunity to tuberculosis, *Annu Rev Immunol,* **22,** 599-623.

O'Connell, R. M., Saha, S. K., Vaidya, S. A., Bruhn, K. W., Miranda, G. A., Zarnegar, B., Perry, A. K., Nguyen, B. O., Lane, T. F., Taniguchi, T., Miller, J. F. and Cheng, G. (2004), Type I interferon production enhances susceptibility to Listeria monocytogenes infection, *J Exp Med,* **200,** 437-445.

O'Garra, A. (1998), Cytokines induce the development of functionally heterogeneous T helper cell subsets, *Immunity,* **8,** 275-283.

O'Garra, A., Vieira, P. L., Vieira, P. and Goldfeld, A. E. (2004), IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage, *J Clin Invest,* **114,** 1372-1378.

Ochando, J. C., Homma, C., Yang, Y., Hidalgo, A., Garin, A., Tacke, F., Angeli, V., Li, Y., Boros, P., Ding, Y., Jessberger, R., Trinchieri, G., Lira, S. A., Randolph, G. J. and Bromberg, J. S. (2006), Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts, *Nat Immunol,* **7,** 652-662.

Orme, I. M., Roberts, A. D., Griffin, J. P. and Abrams, J. S. (1993), Cytokine secretion by CD4 T lymphocytes acquired in response to Mycobacterium tuberculosis infection, *J Immunol,* **151,** 518-525.

Papiernik, M., do Carmo Leite-de-Moraes, M., Pontoux, C., Joret, A. M., Rocha, B., Penit, C. and Dy, M. (1997), T cell deletion induced by chronic infection with mouse mammary tumor virus spares a CD25-positive, IL-10-producing T cell population with infectious capacity, *J Immunol,* **158,** 4642-4653.

Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q. and Dong, C. (2005), A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17, *Nat Immunol,* **6,** 1133-1141.

Portnoy, D. A., Auerbuch, V. and Glomski, I. J. (2002), The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity, *J Cell Biol,* **158,** 409-414.

Powrie, F., Carlino, J., Leach, M. W., Mauze, S. and Coffman, R. L. (1996), A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells, *J Exp Med,* **183,** 2669-2674.

Pym, A. S., Brodin, P., Brosch, R., Huerre, M. and Cole, S. T. (2002), Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti, *Mol Microbiol,* **46,** 709-717.

Quinn, K. M., McHugh, R. S., Rich, F. J., Goldsack, L. M., de Lisle, G. W., Buddle, B. M., Delahunt, B. and Kirman, J. R. (2006), Inactivation of CD4(+)CD25(+) regulatory T cells during early mycobacterial infection increases cytokine production but does not affect pathogen load, *Immunol Cell Biol,* **84,** 467-474.

Read, S., Greenwald, R., Izcue, A., Robinson, N., Mandelbrot, D., Francisco, L., Sharpe, A. H. and Powrie, F. (2006), Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo, *J Immunol,* **177,** 4376-4383.

Read, S., Malmstrom, V. and Powrie, F. (2000), Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation, *J Exp Med,* **192,** 295-302.

Reiling, N., Holscher, C., Fehrenbach, A., Kroger, S., Kirschning, C. J., Goyert, S. and Ehlers, S. (2002), Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis, *J Immunol,* **169,** 3480-3484.

Reis e Sousa, C., Yap, G., Schulz, O., Rogers, N., Schito, M., Aliberti, J., Hieny, S. and Sher, A. (1999), Paralysis of dendritic cell IL-12 production by microbial products prevents infection-induced immunopathology, *Immunity,* **11,** 637-647.

Remus, N., El Baghdadi, J., Fieschi, C., Feinberg, J., Quintin, T., Chentoufi, M., Schurr, E., Benslimane, A., Casanova, J. L. and Abel, L. (2004), Association of IL12RB1 polymorphisms with pulmonary tuberculosis in adults in Morocco, *J Infect Dis,* **190,** 580-587.

Ribeiro-Rodrigues, R., Resende Co, T., Rojas, R., Toossi, Z., Dietze, R., Boom, W. H., Maciel, E. and Hirsch, C. S. (2006), A role for CD4+CD25+ T cells in regulation of the immune response during human tuberculosis, *Clin Exp Immunol,* **144,** 25-34.

Roach, D. R., Briscoe, H., Saunders, B., France, M. P., Riminton, S. and Britton, W. J. (2001a), Secreted lymphotoxin-alpha is essential for the control of an intracellular bacterial infection, *J Exp Med,* **193,** 239-246.

Roach, D. R., Martin, E., Bean, A. G., Rennick, D. M., Briscoe, H. and Britton, W. J. (2001b), Endogenous inhibition of antimycobacterial immunity by IL-10 varies between mycobacterial species, *Scand J Immunol,* **54,** 163-170.

Robinson, D. S. and O'Garra, A. (2002), Further checkpoints in Th1 development, *Immunity,* **16,** 755-758.

Rogers, N. C., Slack, E. C., Edwards, A. D., Nolte, M. A., Schulz, O., Schweighoffer, E., Williams, D. L., Gordon, S., Tybulewicz, V. L., Brown, G. D. and Reis e Sousa, C. (2005), Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins, *Immunity,* **22,** 507-517.

Rojas, R. E., Balaji, K. N., Subramanian, A. and Boom, W. H. (1999), Regulation of human CD4(+) alphabeta T-cell-receptor-positive (TCR(+)) and gammadelta TCR(+) T-cell responses to Mycobacterium tuberculosis by interleukin-10 and transforming growth factor beta, *Infect Immun,* **67,** 6461-6472.

Rolph, M. S., Raupach, B., Kobernick, H. H., Collins, H. L., Perarnau, B., Lemonnier, F. A. and Kaufmann, S. H. (2001), MHC class Ia-restricted T cells partially account for beta2-microglobulin-dependent resistance to Mycobacterium tuberculosis, *Eur J Immunol,* **31,** 1944-1949.

Ronnblom, L., Forsgren, A. and Alm, G. V. (1983), Characterization of interferons induced by bacteria and interferon-producing leukocytes in human peripheral blood, *Infect Immun,* **40,** 126-132.

Sacks, D. (2006) In *Keystone Symposia - Determinants of host resistance, susceptibility or immunopathology to pathogens: Intergrating knowledge from experimental models to human disease.*Keystone, Colorado, USA.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M. (1995), Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases, *J Immunol,* **155,** 1151-1164.

Sakaguchi, S., Setoguchi, R., Yagi, H. and Nomura, T. (2006), Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in self-tolerance and autoimmune disease, *Curr Top Microbiol Immunol,* **305,** 51-66.

Scanga, C. A., Mohan, V. P., Joseph, H., Yu, K., Chan, J. and Flynn, J. L. (1999), Reactivation of latent tuberculosis: variations on the Cornell murine model, *Infect Immun,* **67,** 4531-4538.

Scanga, C. A., Mohan, V. P., Yu, K., Joseph, H., Tanaka, K., Chan, J. and Flynn, J. L. (2000), Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2, *J Exp Med,* **192,** 347-358.

Schaible, U. E., Winau, F., Sieling, P. A., Fischer, K., Collins, H. L., Hagens, K., Modlin, R. L., Brinkmann, V. and Kaufmann, S. H. (2003), Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis, *Nat Med,* **9,** 1039-1046.

Schlesinger, L. S. (1993), Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors, *J Immunol,* **150,** 2920-2930.

Sher, A. and Coffman, R. L. (1992), Regulation of immunity to parasites by T cells and T cell-derived cytokines., *Ann. Rev. Immunol.,* **10,** 385-409.

Shevach, E. M. (2006), From vanilla to 28 flavors: multiple varieties of T regulatory cells, *Immunity,* **25,** 195-201.

Shi, S., Nathan, C., Schnappinger, D., Drenkow, J., Fuortes, M., Block, E., Ding, A., Gingeras, T. R., Schoolnik, G., Akira, S., Takeda, K. and Ehrt, S. (2003), MyD88 primes macrophages for full-scale activation by interferon-gamma yet mediates few responses to Mycobacterium tuberculosis, *J Exp Med,* **198,** 987-997.

Shiloh, M. U. and Nathan, C. F. (2000), Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria, *Curr Opin Microbiol,* **3,** 35-42.

Shortman, K. and Liu, Y. J. (2002), Mouse and human dendritic cell subtypes, *Nature Rev Immunol,* **2,** 151-161.

Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N. and Doetschman, T. (1992), Targeted disruption of the mouse transforming growth factor-b1 gene results in multifocal inflammatory disease., *Nature,* **359,** 693-699.

Silk, J. D., Hermans, I. F., Gileadi, U., Chong, T. W., Shepherd, D., Salio, M., Mathew, B., Schmidt, R. R., Lunt, S. J., Williams, K. J., Stratford, I. J., Harris, A. L. and Cerundolo, V. (2004), Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy, *J Clin Invest,* **114,** 1800-1811.

Silva, R. A. and Appelberg, R. (2001a), Blocking the receptor for interleukin 10 protects mice from lethal listeriosis, *Antimicrob Agents Chemother,* **45,** 1312-1314.

Silva, R. A., Pais, T. F. and Appelberg, R. (2001b), Blocking the receptor for IL-10 improves antimycobacterial chemotherapy and vaccination, *J Immunol,* **167,** 1535-1541.

Sousa, A. O., Mazzaccaro, R. J., Russell, R. G., Lee, F. K., Turner, O. C., Hong, S., Van Kaer, L. and Bloom, B. R. (2000), Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice, *Proc Natl Acad Sci U S A,* **97,** 4204-4208.

Steinman, R. M. (1991), The dendritic cell system and its role in immunogenicity, *Ann. Rev. Immunol.,* **9,** 271-296.

Sturgill-Koszycki, S., Schaible, U. E. and Russell, D. G. (1996), Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis, *Embo J,* **15,** 6960-6968.

Sundstedt, A., O'Neill, E. J., Nicolson, K. S. and Wraith, D. C. (2003), Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo, *J Immunol,* **170,** 1240-1248.

Suvas, S., Kumaraguru, U., Pack, C. D., Lee, S. and Rouse, B. T. (2003), CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses, *J Exp Med,* **198,** 889-901.

Suzuki, Y., Sher, A., Yap, G., Park, D., Neyer, L. E., Liesenfeld, O., Fort, M., Kang, H. and Gufwoli, E. (2000), IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and

susceptible C57BL/6 mice following peroral infection with Toxoplasma gondii, *J Immunol,* **164,** 5375-5382.

Tailleux, L., Neyrolles, O., Honore-Bouakline, S., Perret, E., Sanchez, F., Abastado, J. P., Lagrange, P. H., Gluckman, J. C., Rosenzwajg, M. and Herrmann, J. L. (2003), Constrained intracellular survival of Mycobacterium tuberculosis in human dendritic cells, *J Immunol,* **170,** 1939-1948.

Takeda, K. and Akira, S. (2005), Toll-like receptors in innate immunity, *Int Immunol,* **17,** 1-14.

Taylor, P. R., Martinez-Pomares, L., Stacey, M., Lin, H. H., Brown, G. D. and Gordon, S. (2005), Macrophage receptors and immune recognition, *Annu Rev Immunol,* **23,** 901-944.

Thornton, A. M. and Shevach, E. M. (1998), CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production, *J Exp Med,* **188,** 287-296.

Tian, T., Woodworth, J., Skold, M. and Behar, S. M. (2005), In vivo depletion of CD11c+ cells delays the CD4+ T cell response to Mycobacterium tuberculosis and exacerbates the outcome of infection, *J Immunol,* **175,** 3268-3272.

Trinchieri, G. (2003), Interleukin-12 and the regulation of innate resistance and adaptive immunity, *Nat Rev Immunol,* **3,** 133-146.

Turner, J., Gonzalez-Juarrero, M., Ellis, D. L., Basaraba, R. J., Kipnis, A., Orme, I. M. and Cooper, A. M. (2002), In vivo IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 mice, *J Immunol,* **169,** 6343-6351.

Ulrichs, T. and Kaufmann, S. H. (2006), New insights into the function of granulomas in human tuberculosis, *J Pathol,* **208,** 261-269.

Ulrichs, T., Kosmiadi, G. A., Trusov, V., Jorg, S., Pradl, L., Titukhina, M., Mishenko, V., Gushina, N. and Kaufmann, S. H. (2004), Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung, *J Pathol,* **204,** 217-228.

Underhill, D. M., Ozinsky, A., Smith, K. D. and Aderem, A. (1999), Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages, *Proc Natl Acad Sci U S A,* **96,** 14459-14463.

van Pinxteren, L. A., Cassidy, J. P., Smedegaard, B. H., Agger, E. M. and Andersen, P. (2000), Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells, *Eur J Immunol,* **30,** 3689-3698.

Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. and Stockinger, B. (2006), TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells, *Immunity,* **24,** 179-189.

Vieira, P. L., Christensen, J. R., Minaee, S., O'Neill, E. J., Barrat, F. J., Boonstra, A., Barthlott, T., Stockinger, B., Wraith, D. C. and O'Garra, A. (2004), IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells, *J Immunol,* **172,** 5986-5993.

Vordermeier, H. M., Venkataprasad, N., Harris, D. P. and Ivanyi, J. (1996), Increase of tuberculous infection in the organs of B cell-deficient mice, *Clin Exp Immunol,* **106,** 312-316.

Wagner, R. D., Maroushek, N. M., Brown, J. F. and Czuprynski, C. J. (1994), Treatment with anti-interleukin-10 monoclonal antibody enhances early resistance to but impairs complete clearance of Listeria monocytogenes infection in mice, *Infect Immun,* **62,** 2345-2353.

Wangoo, A., Sparer, T., Brown, I. N., Snewin, V. A., Janssen, R., Thole, J., Cook, H. T., Shaw, R. J. and Young, D. B. (2001), Contribution of Th1 and Th2 cells to protection and pathology in experimental models of granulomatous lung disease, *J Immunol,* **166,** 3432-3439.

Whelan, M., Harnett, M. M., Houston, K. M., Patel, V., Harnett, W. and Rigley, K. P. (2000), A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells, *J Immunol,* **164,** 6453-6460.

WHO (1993), Tuberculosis: a global emergency, *World Health Forum,* **14,** 438.

WHO (2006) *Global Tuberculosis Control - Surveillance, Planning, Financing.*

Winau, F., Kaufmann, S. H. and Schaible, U. E. (2004a), Apoptosis paves the detour path for CD8 T cell activation against intracellular bacteria, *Cell Microbiol,* **6,** 599-607.

Winau, F., Schwierzeck, V., Hurwitz, R., Remmel, N., Sieling, P. A., Modlin, R. L., Porcelli, S. A., Brinkmann, V., Sugita, M., Sandhoff, K., Kaufmann, S. H. and Schaible, U. E. (2004b), Saposin C is required for lipid presentation by human CD1b, *Nat Immunol,* **5,** 169-174.

Wozniak, T. M., Ryan, A. A., Triccas, J. A. and Britton, W. J. (2006), Plasmid interleukin-23 (IL-23), but not plasmid IL-27, enhances the protective efficacy of a DNA vaccine against Mycobacterium tuberculosis infection, *Infect Immun,* **74,** 557-565.

Wright, A. (2006), Emergence of Mycobacterium tuberculosis With Extensive Resistance to Second-Line Drugs - Worldwide, 2000-2004., *JAMA,* **295,** 2349-2351.

Xu, D., Liu, H., Komai-Koma, M., Campbell, C., McSharry, C., Alexander, J. and Liew, F. Y. (2003), CD4+CD25+ regulatory T cells suppress differentiation and functions of Th1 and Th2 cells, Leishmania major infection, and colitis in mice, *J Immunol,* **170,** 394-399.

Young, D. and Dye, C. (2006), The development and impact of tuberculosis vaccines, *Cell,* **124,** 683-687.

Young, D. B. (2003), Ten years of research progress and what's to come, *Tuberculosis (Edinb),* **83,** 77-81.

Appendices:

Appendix I: Keystone Symposium poster presentation (Jan. 2006): "Determinants of host resistance, susceptibility or immunopathology to pathogens"

The role of plasmacytoid precursor dendritic cells in anti-mycobacterial immunity.

Paul Redford[1], Andre Boonstra[1], Mary Holman[1], Giorgio Trinchieri, Greg Bancroft[2] and Anne O' Garra[1].
[1]Division of Immunoregulation, National Institute for Medical Research (NIMR), London, NW7 1AA, United Kingdom.
[2]Dept. of Immunology, London School of Hygiene and Tropical Medicine, London, UK.

Plasmacytoid precursor dendritic cells (pDC) play a pivotal role in anti-viral immune responses through the production of large quantities of type I interferon (primarily IFN-alpha). Numerous studies to date have focused on the role of plasmacytoid pDC during viral infections; however, the relative contribution of this DC subset during bacterial infections is largely unknown. We have previously reported that plasmacytoid pDC activated *in vitro* with un-methylated CpG DNA produce high levels of IL-12p70 and IFN-alpha, whilst inducing strong Th1 responses. IL-12 is well known to promote protective Th1 responses, and its production can be inhibited under certain conditions, by IFN-alpha. In line with this, recent studies have shown enhanced immune responses to *Listeria monocytogenes* in the absence of IFN-alpha/beta signalling. The balance of IL-12 versus IFN-alpha during infections may therefore be important for the outcome of the immune response.

The immune response to *Mycobacterium tuberculosis* (MTb) strongly depends on protective Th1 responses, in which IFN-gamma and TNF play pivotal roles. To date, there is little published data on plasmacytoid pDC or type I IFN during *in vivo* MTb infection. One study has shown the presence of plasmacytoid pDC in human MTb infected tonsil tissue. Another study in a murine model of MTb, has postulated that the hyper-virulent phenotype of the clinical isolate HN878 in mice is due to induction of type I IFN. To examine whether Th1 responses during MTb infection are suboptimal due to the inhibitory effect of IFN-alpha we studied the role of plasmacytoid pDC during MTb infection in mice. *In vitro* MTb infection of bone-marrow-derived myeloid DC resulted in the production of IL-12p40, IL-10 and TNF, as previously reported by others. In contrast, *in vitro* MTb infection of freshly isolated plasmacytoid pDC from the spleen did not produce detectable levels of the cytokines IL-12p40, TNF, IL-10 or IFN-alpha. Other markers of plasmacytoid pDC activation following *in vitro* MTb infection are under investigation. Currently, we are assessing the role of plasmacytoid pDC *in vivo* by studying the potential influx of this DC subset into sites of MTb infection, their activation state, as well as the effect of specific depletion of plasmacytoid pDC. Determining the role of plasmacytoid pDC during MTb infection will further our understanding of the role of type I IFN in the immunology of MTb and in addition may highlight important implications for future vaccine design.

Appendix II: Keystone Symposium poster presentation (March 2007): "Tuberculosis: from lab research to field trials"

Regulatory mechanisms inhibiting anti-mycobacterial immunity following *Mycobacterium tuberculosis* infection:

Paul Redford[1], André Boonstra[1,2], Simon Read[1], Mary Holman[1], Gregory Bancroft[3] and Anne O' Garra[1].

[1] Division of Immunoregulation, National Institute for Medical Research, London, UK.

[2] Dept. Gastroenterology & Hepatology, Erasmus Med. Center, Rotterdam, Netherlands.

[3] Dept. of Immunology, London School of Hygiene and Tropical Medicine, London, UK.

Control and clearance of intracellular pathogens such as *M. tuberculosis* (MTb) is dependent on TNF and the induction of the T-helper 1 (Th1) cytokine IFN-gamma by IL-12. Conversely, IL-10 is a suppressive cytokine essential for dampening the immune response to a number of intracellular pathogens to limit host immune pathology. During infection with pathogens such as *T. gondii,* IL-10 prevents pathogen clearance but is critical for blocking immune pathology. In patients with chronic tuberculosis (TB), T cells are present that produce both IL-10 and IFN-gamma and stimulation of MTb-antigen specific T cells *ex-vivo* in the presence of anti-IL-10 antibodies results in their increased proliferation and IFN-gamma production. Although this suggests a role for IL-10 in the regulation of the response to MTb, this has not been demonstrated and it is possible that a major role at this stage is to limit immune pathology in chronic disease.

To date in murine models of MTb, a role for IL-10 is controversial. To determine a regulatory role for IL-10 following murine infection, we examined its function during both acute and chronic stages of infection with the MTb strain: H37Rv obtained from the London School of Hygiene and Tropical Medicine (LSHTM). IL-10 receptor blockade during the chronic phase of MTb infection with H37Rv (LSHTM) did not affect bacterial levels, even when the number of bacilli used to infect mice was decreased. Despite the lack of effect of IL-10 blockade on the bacterial load, during chronic infection with H37Rv (LSHTM), immune cells obtained from MTb infected mice produced elevated levels of IFN-gamma when stimulated *in vitro* in the presence of anti-IL-10R antibodies. Most importantly, neutralisation of IL-10 before and during acute MTb infection with H37Rv (LSHTM) resulted in a transient reduction in bacteraemia and enhanced IFN-gamma production, suggesting that IL-10 plays a role in regulating the immune response to MTb at a very early stage of the innate immune response. With these findings in mind, we hypothesise that IL-10 plays a role to limit the early protective response to MTb, but may function later to contain the immune response and inhibit host pathology, which we are currently investigating.